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LARISSA MARTINS MELO

O PAPEL DOS MIRNA NA INFECÇÃO DE LEUCÓCITOS ESPLÊNICOS DE CÃO POR *LEISHMANIA INFANTUM*

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Tese apresentada à Faculdade de Medicina Veterinária de Araçatuba da Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP, como parte dos requisitos para a obtenção do título de Doutora em Ciência Animal (Área de Medicina Veterinária Preventiva e Produção Animal).

Orientadora: Prof.ª Ass. Valéria Marçal Felix de Lima

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Melo, L.M. et al. **O papel dos mirna na infecção de leucócitos esplênicos de cão por *Leishmania Infantum.*** 2018, 72 f. Tese (Doutorado) - Faculdade de Medicina Veterinária, Universidade Estadual Paulista, Araçatuba, 2018.

RESUMO

A leishmaniose visceral canina (LV) no Brasil representa um grave problema de saúde pública. Na LV a supressão imune celular é determinante da progressão da doença. Estudos mostraram que a regulação da resposta imune depende de miRNAs. O objetivo deste estudo foi a caracterização dos miRNAs em leucócitos esplênicos (LE) de cães naturalmente infectados por *L. infantum*. Este estudo foi realizado em Araçatuba, região endêmica para LV canina (LVC). Um grupo de 4 cães saudáveis e 8 cães com LVC foi estudado. O RNA foi extraído do LE usando o Kit Mirvana (Invitrogen™) de acordo com as recomendações do fabricante. O RNA foi quantificado usando um fluorômetro (Qubit 3.0, Invitrogen™) o grau de pureza realizado por eletroforese capilar (Bioanalyser, Agilent ™), as amostras foram então armazenadas a -80°C. O miRNA foi preparado para o microarranjo usando o FlashTag Biotina HSR RNA Labeling Kit (Affymetrix ™). O microarranjo foi realizado usando o Affymetrix ™ miRNA 4.1 Strip de acordo com as recomendações do fabricante. A produção de cDNA foi realizada usando o kit miScript RT II (Qiagen™). Os miRNAs diferencialmente expressos foram validados por qPCR utilizando iniciadores para miRNAs de cães inventariados (Qiagen™) de acordo com recomendação do fabricante. Os miRNAs validados foram analisados no programa Ingenuity Pathway Analysis (IPA, Qiagen™). Após a análise das vias, os LE de cães infectados foram transfectados com miR21 usando miScript miRNA Mimics e Inhibitor (Qiagen™) de acordo com as recomendações do fabricante. Após a transfecção, o factor de transcrição T-bet e GATA 3 e a carga parasitária foi avaliada por citometria de fluxo e a IL-12 foi quantificada por ELISA Kit (R & D Systems™). A análise do microarray foi realizada no Expression Console, no Transcriptome Analysis Console (Affymetrix™) por ANOVA, qPCR foi analisada pelo teste de Mann-Whitney, a via canônica de miRNA diferentemente expressa em IPA com o teste de Fisher, o fator de transcrição, carga parasitária e expressão de IL-12 foi analisada pelo teste de Friedman, foi utilizado o nível de significância de $p<0,05$. Verificou-se que 7 miRNAs tiveram

expressão alterada em cães infectados em comparação com cães saudáveis: miR-148a, miR-21, miR-7, miR-615 foram “upregulated” e miR-150, miR-125a e miR-125b foram “downregulated”. O miR148a, miR615 e miR21 validaram os resultados do microarranjo. O IPA mostrou 114 vias reguladas por esses miRNAs, incluindo vias que regulam a imunidade. Após a transfecção inibimos o miRNA 21 e observamos um aumento de IL-12, da razão Th1 e Th2 e uma diminuição da carga parasitária. Concluímos, que o miR 21 interfere na resposta imunológica de cães infectados por *L. infantum*, inibindo a resposta do tipo celular. Essas informações podem ajudar na identificação de alvos terapêuticos na LVC.

Palavras-chave: *Leishmania (L.) infantum*, miRNA, transfecção.

Melo, L.M. et al. **The role of mirna in the infection of dog spleen leukocytes by *Leishmania Infantum.*** 2018, 72 f. Tese (Doutorado) - Faculdade de Medicina Veterinária, Universidade Estadual Paulista, Araçatuba, 2018.

ABSTRACT

Canine visceral leishmaniasis (VL) in Brazil represents a serious public health problem. In VL cellular immune suppression is determinant of disease progression. Studies have shown that the regulation of the immune response depends on miRNAs. The objective of this study was the characterization of the miRNAs in spleen leukocytes (SL) from dogs naturally infected by *L. infantum*. This study was carried out in Araçatuba, an endemic region for canine LV (LVC). A group of 4 healthy dogs and 8 dogs with VL was studied. Total RNA was extracted from SL using Mirvana Kit (Invitrogen®) according to manufacturer's recommendations Total RNA was quantified using a fluorometer (Qubit 3.0, Invitrogen®) the degree of purity performed by capillary electrophoresis (Bioanalyser, Agilent®), the samples were then stored at -80°C. Total RNA was prepared for the microarray using the FlashTag Biotin HSR RNA Labeling Kit (Affymetrix®). The microarray was performed using the Affymetrix ™ miRNA 4.1 Strip according to manufacturer's recommendations. cDNA production was performed using the miScript RTII kit (Qiagen®). Differentially expressed miRNAs were validated by qPCR was performed using the inventoried dog miRNAs (Qiagen®) and SYBR Green (kit miScript SYBR Green PCR, Qiagen®), according to the manufacturer's recommendation. The validated miRNAs were analyzed in the program Ingenuity Pathway Analysis (Qiagen®). After analysis of the pathways, then the LS from infected dogs were transfected with miR21 using miScript miRNA Mimics and Inhibitor (Qiagen®) according to manufacturer's recommendations. After the transfection LE the transcription factor T-bet and GATA 3, parasite load were measured by flow cytometry and IL-12 was quantified by ELISA Kit (R&D Systems®). The analyses of microarray was performed on Expression Console, Transcriptome Analysis Console (Affymetrix®); ANOVA, qPCR validation data was analyzed by Mann-Whitney test, the canonical pathway of miRNA differentially expressed in IPA with the Fisher test, the transcription factor, parasite load and expression of IL-12 was analysed by Friedman test, with the level of significance of $p < 0.05$. It was found that 7 miRNAs had altered

expression in infected dogs compared to healthy dogs: miR148a, miR21, miR7, miR615 were upregulated and miR150, miR125a and miR125b were downregulated. MiR148a, miR615 and miR21 validated the results of the microarray. The IPA showed 114 pathways regulated by these miRNAs, including pathways which regulate immunity. After transfection, the miR21 inhibitors increased T-bet and IL12 and decreased parasitic load. We conclude that miR 21 interferes in the immune response of dogs infected with L. infantum, inhibiting the cell type response. This information can assist in the identification of therapeutic targets in LVC.
Keywords: *Leishmania (L.) infantum*, miRNA, transfection.

SUMÁRIO

1. CAPÍTULO I

1.1. Introdução geral

A leishmaniose é uma doença antropo-zoonótica causada por várias espécies de *Leishmania* pertencentes aos subgêneros *Leishmania* e *Viannia*. Afetando cerca de 12 milhões de pessoas, é considerado um problema de saúde global devido à sua difusão na Europa, África, Ásia (Velho Mundo), bem como nas Américas (Novo Mundo) (AKHOUNDI et al., 2016; ALVAR et al., 2012). As leishmanioses ocorrem em 88 países, dos quais 65 apresentam a forma visceral. A maioria dos casos (90%) de Leishmaniose Visceral (LV) em humanos ocorrem em áreas rurais ou suburbanas de cinco países, incluindo o Brasil, que com a migração para as áreas urbanas contribuiu para a expansão da LV (DESJEUX, 2004). Estima-se que haja 50 a 90 mil novos casos de LV todos os anos (WORLD HEALTH ORGANIZATION, 2018)

No Brasil, essa expansão ocorreu principalmente no Nordeste (92%) (DANTAS-TORRES, 2006) e áreas do Sudeste (4%) (SANTIAGO et al., 2013), do Norte (3%) e do Centro Oeste (1%). Além da alta incidência e ampla distribuição, a expansão em novas áreas também carrega a ameaça de que as formas graves e letais da doença possam surgir quando associada à desnutrição (GONTIJO; MELO, 2004).

Na LV os principais reservatórios fora do ambiente silvestre, os cães são importantes na manutenção do ciclo epidemiológico da doença porque: a) LV é mais prevalente na população canina que na humana; b) a infecção no homem normalmente é precedida por casos caninos; c) cães apresentam maior quantidade de parasitos na pele do que o homem, fato que favorece a infecção dos vetores (SANTA ROSA E OLIVEIRA, 1997). A prevalência de leishmaniose em cães de áreas endêmicas pode atingir de 20 a 40% da população (SLAPPENDEL;FERRER, 1990).

Estudos revelam que a LV é a mais severa das leishmanioses, podendo ser fatal quando não tratada adequadamente, pois atinge órgãos como baço, linfonodos, fígado e medula óssea, que possuem macrófagos em sua constituição (REIS et al., 2009; STRAUSS-AYALI; BANETH; JAFFE, 2007). Uma outra característica particular da doença é a ineficácia do tratamento quando o animal já se encontra infectado. Além disso, alguns animais comportam-se como portadores assintomáticos da doença, cuja evolução de caráter crônico impossibilita a correta

identificação dos doentes (REIS et al., 2009; STRAUSS-AYALI; BANETH; JAFFE, 2007).

Os cães são considerados os principais reservatórios domésticos de *L. chagasi* (MORENO; ALVAR, 2002). O parasita é transmitido de um cão infectado para outro através da picada de flebotomíneos e possivelmente, através de outros artrópodes vetores, como pulgas (FERREIRA et al., 2009) e carrapatos (COUTINHO et al., 2005; COUTINHO; LINARDI, 2007), e também através de transfusões de sangue (OWENS et al., 2001). Durante o nepasto sanguineo de flebotomíneos em cães, os parasitas são inoculados na pele e rapidamente se espalham para os linfonodos e o baço através da linfa e sangue e, eventualmente, atingem também os rins e o fígado. Órgãos reprodutores, bexiga, o sistema digestivo, a pele e o sistema respiratório (MOLYNEUX; ASHFORD, 1983).

Uma vez no hospedeiro vertebrado, o parasita pode causar sinais clinicos que são característicos de Leishmaniose Visceral Canina (LVC). Alguns cães podem apresentar poucos ou nenhum sinal clinico (MANCIANTI et al., 1988), ou mesmo evoluir para a cura espontânea (FISA et al., 1999; GENARO, 1993). Os sinais clínicos mais frequentes de LVC são linfadenomegalia, onicogrifose, lesões cutâneas, perda de peso, caquexia e anormalidades locomotoras (SEMIÃO-SANTOS et al., 1995). A forma assintomática representa de 20 a 40% da população de soro-positivos, dos quais 80%, desenvolvem a doença de fato (NOLI; BOOTHE, 1999). No Brasil, em área urbana da região nordeste, a forma assintomática representa 30% da população soro positivo (QUEIROZ et al., 2009).

A supressão da imunidade celular é o aspecto mais importante na patogênese e progressão da doença canina. A ausência de resposta das células T aos antigenos de *Leishmania* sp. é observada in vivo, com um teste de Montenegro negativo (DOS-SANTOS et al., 2008). Em cães infectados com *Leishmania infantum*, ocorre uma redução do número de linfócitos T em sangue periférico (BOURDOISEAU et al., 1997; DE LIMA et al., 2012) e desorganização da polpa branca do baço (SANTANA et al., 2008).

A imunidade protetora tem geralmente sido associada com resposta imunológica celular, manifestada por resposta linfoproliferativa positiva a antígenos de *Leishmania* sp. (CABRAL; O'GRADY; ALEXANDER, 1992) e produção de citocinas, tais como IFN-gama e TNF-alfa , as quais são necessárias para ativação e macrófagos e morte de parasitas intracelulares (SADICK et al., 1991).

Os macrófagos têm papel importante na resposta inata e adaptativa imunológica contra *Leishmania* sp, pois são células parasitadas e além disso iniciam a resposta adaptativa. A célula T responde ao antígeno através do engajamento do seu receptor TCR ao MHC associado ao peptídeo antigênico no macrófago. A função efetora desta interação é determinada por sinais adicionais da ligação de moléculas coestimulatórias sobre a superfície de células T e seus ligantes nas células apresentadoras de antígeno. Dependendo da natureza e magnitude desses sinais a célula irá gerar citocinas ou desenvolver funções regulatórias ou citotóxicas, indução de memória ou anergia (PENTCHEVA-HOANG et al., 2007).

As moléculas coestimulatórias pertencem a família B7-CD28, as quais transmitem sinais ativadores (CD28) ou inibidores (CTLA-4) para as células T (GREENWALD; FREEMAN; SHARPE, 2005). Em macrófagos infectados por *Leishmania* sp ocorre falha no processo de apresentação de antígenos e a análise da expressão das moléculas coestimulatórias indicam diminuição na expressão de B7; que reduz a proliferação das células T (PINELLI et al., 1999). Tem sido recentemente mostrado que o parasita e seus antígenos inibem a expressão de co-estimuladores e diminuem a produção de óxido nítrico (DIAZ et al., 2012).

A resposta imune protetora contra *Leishmania* em cães está relacionada ao aumento da resposta T1 com a produção de IFN-γ e lL-2, que são citocinas que promovem ativação de macrófagos e de linfócitos citotóxicos (PINELLI et al., 1994, 1995). A eliminação do parasito em macrófagos depende da produção de óxido nítrico mediada pela enzima óxido nítrico sintetase induzível (iNOS), sendo esta produzida por IL-12 e TNF-α (BELOSEVIC et al., 1990)

A infecção por *Leishmania* em sangue periférico de pacientes aumenta a produção de IL-10, enquanto diminui a produção de IL-12 (CILLARI et al., 1991). A *Leishmania* modula a expressão de citocinas (principalmente IL-10 e IL-12) diferencialmente em seu próprio favor, THP-1 (linhagem celular de humanos) infectados por *L. donovani*, mostrou um aumento na IL-10 e uma diminuição na produção de IL-12 (HUSEIN et al., 2018).

Após a infecção por *Leishmania*, a capacidade das células dendriticas de camundongos em produzir IL-12 é extremamente importante para a polarização de células T e subsequente produção de IFN-γ (FEIJÓ et al., 2016). Na infecção com *L. donovani* de cães in vivo e ex vivo a resposta imunológica capaz de combater o parasita foi associada à produção de anticorpos e respostas proliferativas CD4 (+) e

CD8 (+), como também à ativação de células T e produção de IFN-γ, IL-12 / IL-23p40 e TNF-α por células mononucleares de sangue periférico (FIUZA et al., 2015). Em células esplênicas de cães infectados com *L. infantum* foi observada uma correlação negativa da carga parasitária com as citocinas pró-inflamatórias: IFNγ, IL-12, IL-6; e com as citocinas anti-inflamatórias: IL-10 e TGF-β (CAVALCANTI et al., 2015).

A função da célula T na indução da resposta celular é determinante para a eliminação do parasita no interior dos macrófagos (PINELLI et al., 1999). A regulação da função efetora dos macrófagos e células T parece depender de pequenos RNAs não codificantes de aproximadamente 21 nucleotídeos de comprimento e que são transcritos no interior do núcleo celular e funcionam como reguladores pós-transcricionais de expressão gênica, regulando a tradução de proteínas fundamentais para a resposta imunológica. Essas moléculas são denominadas de microRNA (miRNA). Durante os últimos anos essas moléculas foram evidenciadas por apresentarem um papel crítico no desenvolvimento e função da resposta imunológica (LIU; ABRAHAM, 2013). Os macrófagos passam por mudanças dramáticas na expressão dos genes, resultando em alterações na capacidade funcional da célula. Os miRNAs tem a capacidade de atingir simultaneamente centenas de RNA mensageiro (mRNA) transcritos, e promover a diferenciação em macrófagos com um fenótipo inflamatório ou não (EL GAZZAR et al., 2011; GRAFF et al., 2012).

Os miRNAs também estão envolvidos na resposta adaptativa regulando a ativação, proliferação e apoptose das células T e B. Na leishmaniose visceral a expressão diferencial de miRNAs já foi observada em diferentes modelos regulando a imunidade. A indução do miR-346 em células de linhagem humanas infectadas com *L. infantum* e *L. Viannia*, na análise in silico mostrou que o miRNA 346 pode desempenhar um papel na regulação das funções macrofágicas, já que genes associados ao interferon ou MHC estão entre os alvos deste miRNA (DIOTALLEVI et al., 2018)

Os macrófagos de camundongos infectados por *Leishmania donovani* mostraram expressão diferencial de 10 miRNAs (mir-3620, mir-6385, mir-6973a, mir-6996, mir-328, mir-8113, mir-3473f, mir-763, mir-6540, e mir -1264) e análises in silico revelaram o envolvimento desses miRNAs em vários processos biológicos, como inibição da apoptose, fagocitose, resposta a drogas e transições fenotípicas de células T (TIWARI et al., 2017).

Células dendríticas e macrófagos humanos infectados in vitro com *L. donovani* apresentaram diminuição a expressão dos microRNAs: miR-21, mir-155 e miR-146b-5p interferindo principalmente na via de sinalização do TGF-β (GERACI; TAN; MCDOWELL, 2015).

Na infecção experimental em camundongo por *L. donovani* foi observado no fígado uma diminuição do miR-122 e genes envolvidos na biossíntese de colesterol, e a restauração da atividade do miR-122 restabeleceu os níveis de colesterol e reduziu a contagem parasitária (GHOSH et al., 2013). Esse dado sugere que na LV os miRNA tem papel importante e podem estar regulando a resposta imunológica.

Análises in silico de células humanas T2/ Treg foi identificado os miRNAs: miR-135, miR-126 miR-29-b e miR-29ª, o aumento ou administração destes miRNAs pode ser utilizada no desenvolvimento da resposta imunitária T1/TH17 protetora durante a infecção por LV (PANDEY; SUNDAR; PRAJAPATI, 2016). Outro estudo mostrou que a *L. donovani* regula positivamente a expressão de Rab5a em macrófagos humanos inibindo a expressão de miR-494, pois o nocaute de Rab5a ou aumento da expressão do miR 494 inibe significativamente a sobrevivência dos parasitas remodulando o tráfego de vesículas endossomais (VERMA; RASTOGI; MUKHOPADHYAY, 2017). A modulação da produção de IL-12 já vem sendo demonstrada por interferências de miRNAs, como na infecção de macrófagos de camundongos com *Leishmania donovani* resistente a antimônio, mostrou que o miR-466i desativa a via MyD88 no hospedeiro e regula os níveis de IL-10 / IL-12 durante as primeiras horas de infecção, determinando o curso da doença (MUKHERJEE et al., 2015).

Além dos estudos em Leshmania donovani e infantum outros trabalhos emonstraram a importância dos miRNAs na imunologia,

O miRNA 615 que está relacionado a um aumento na capacidade fagocítica de macrófagos esplênicos (JIANG et al., 2011). E no baço de cães com LVC há um aumento na proliferação e diferenciação de macrófagos (ALEXANDRE-PIRES et al., 2006; BANETH et al., 2008).

O miR 21 tem como alvo CD69 (CARISSIMI et al., 2014), marcador de ativação de linfócitos, incluindo células NK (BORREGO et al., 1999; ZHANG et al., 2015). As células NK desempenham um papel fundamental na indução e polarização para Th1 ou Th2. As promastigotas de *Leishmania* podem induzir diretamente as

células NK do sangue periférico humano de doadores saudáveis a secreção de IFN-γ na presença de IL-12 e células apresentadoras de antígenos profissionais (NYLÉN et al., 2003), e isso pode ser relevante para o estabelecimento da resposta imune Th1, que é a resposta celular de proteção na LVC, porém em cães o envolvimento de miR 21 com a função efetora das células NK não está elucidado.

A citocina TNF-α está relacionada a resistência na LVC (CHAMIZO; MORENO; ALVAR, 2005) e tem sua expressão diminuída por miR 21 (YANG et al., 2017; ZHANG et al., 2012) e por miR148a (LIU et al., 2010). Em cães com LVC assintomáticos há uma alta expressão de TNF-α em linfonodos com baixo parasitismo, e essa expressão é menor em cães sintomáticos, com maior carga parasitária, sugerindo que essa citocina possui uma função de proteção na infecção por *L. infantum* (ALVES et al., 2009), porém a associação dessa citocina com miRNA 21e miR 148a na LVC ainda não foi estudada.

A citocina IL-12 tem um papel importante na indução da imunidade inata, estimulando a produção de IFN-ɣ e diferenciação de células Th1 (SALDARRIAGA et al., 2006) e IL-12 é um alvo de miR 21 (LU et al., 2011; LU; MUNITZ; ROTHENBERG, 2009; WU et al., 2012) e também de miR 148a (LIU et al., 2010). Um estudo com cães naturalmente infectados por *L. infantum* mostrou que a IL-12 além de aumentar a proliferação de células mononucleares de sangue periférico, também aumenta a produção de IFN-γ por essas células, o que faz com que ele seja uma boa opção para terapias com citocinas em cães que são refratários a terapia atual (STRAUSS-AYALI et al., 2005).

A sinalização de TCR em linfócitos T de camundongo é diminuída quando tem o aumento da expressão do miR 21 em células Jurkat, diminuindo a fosforalização de ERK, ativação de AP-1 e expressão de CD69 (CARISSIMI et al., 2014). O bloqueio do miR 21 em células mononucleares de camundongos infectados com *L. donovani* (LdWT) permitiu a forte proliferação de células T CD4 (BHATTACHARYA et al., 2017). Estudos explorando os miRNAs como ferramentas terapêuticas estão em andamento para tratamento do vírus da hepatite C e certas formas de câncer (UCHINO; OCHIYA; TAKESHITA, 2013). Na LVC devido a supressão imunológica celular ser determinante da progressão da doença, o conhecimento de fatores associados à regulação imunológica é fundamental para a mudança do padrão de resposta. Assim o objetivo dessa pesquisa é analisar os miRNAs diferencialmente expressos nos LE de cães naturalmente infectados por *L. infantum*, analisar os alvos e vias dos miRNAs

diferencialmente expressos nos LE de cães naturalmente infectados por *L. infantum* e analisar a IL-12 como alvo do miR 21 em LE de cães naturalmente infectados por *L. infantum.*

2. CAPÍTULO 2

INDUCTION OF MIR 21 IMPAIRS THE ANTI-LEISHMANIA RESPONSE TARGETING IL-12

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ABSTRACT

Visceral Leishmaniasis (VL) is a chronic zoonosis and, if left untreated, can be fatal. The infected dogs have decreased cellular immunity (T1) and develop a potent humoral response (T2), which is not effective for elimination of the protozoan. The immune response can be modulated by microRNAs (miRNAs). However, no study of the characterization and regulatory role of miRNAs in the spleen in CVL was performed. We evaluated the miRNAs differentially expressed in splenic leukocytes (SL) from dogs naturally infected with Leishmania (L.) infantum (n = 8) compared to healthy dogs (n = 4). Microarray analysis showed that miRNAs: miR 21, miR 148a, miR 7 and miR 615 showed increased expression, and the miRNAs: miR 150, miR 125a and miR 125b showed decrease in expression. Real-time PCR validated the expression of miR 21, miR 148a and miR 615. The decrease of miR 21 in the LE by the miR 21 inhibitor transfection increased the IL-12 cytokine and the T-bet / GATA-3 ratio and decreased the loading parasite of LE from dogs with CVL.These findings suggest *L. infantum* infection alters the expression of miRNAs and that miR 21 interferes in the cellular immune response of *L. infantum*-infected dogs, helping to identify therapeutic targets in CVL.

Key words: MicroRNAs, Visceral Canine Leishmaniasis, miR 21, immune response, transfection

AUTHOR ABSTRACT

Visceral leishmaniasis is a chronic disease that affects humans, dogs, cats and other mammals. The disease is caused by a protozoan called *Leishmania infantum* and can lead to death if left untreated. Dogs are the main transmitters of the parasite to humans by the phlebotomine vector. Dogs develop an ineffective immune response against this infection, and it can be modulated by small RNAs called microRNAs, which are non-coding RNAs that can regulate gene expression, thereby modulating the immune response. In this study, we investigated the microRNAs involved in canine visceral leishmaniasis, analyzed the target genes that modified the expression of these miRNAs. We analyzed the cytokine IL-12, T1 / T2 profile and the parasite load. These findings may aid in the identification of therapeutic targets for this disease.

1. INTRODUCTION

Visceral Leishmaniasis (VL) is caused by the protozoan *Leishmania (L.) infantum* [1]. This infection was considered one of the most severe forms of the disease [2] and has seen a very significant growth in recent years, being a serious problem in public health [3]. There are 65 countries that present the visceral form, most cases occurring in Brazil, East Africa and Southeast Asia [4]. It is estimated that 50,000 to 90,000 new cases of VL occur worldwide each year [4]. In humans and dogs, the parasite can cause lesions and symptoms that are characteristic of VL [5,6], being the most frequent in dogs: lymphadenopathy, onychogrifosis, cutaneous lesions, weight loss, cachexia and locomotor abnormalities [7].

In canine visceral leishmaniasis the spleen is one of the most affected organs during infection [8] along with skin and bone marrow [9]. High parasitism is observed in the spleen and consequently presents significant morphological changes such as hypertrophy and hyperplasia of the red pulp with infiltration of mononuclear cells and mainly plasma cells [10]. The white pulp presents replacement of macrophages by lymphocytes due to the hypertrophy and hyperplasia of this region [10], unlike peripheral blood, the spleen is the place where the immune response to the parasite will occur with macrophage and lymphocyte activation. The immune response of the dog to the parasite is compartmentalized [11], emphasizing the importance of the study of the spleen.

In VL, protective immunity has been associated with a cellular immune response [12], manifested by positive lymphoproliferative response to *Leishmania* spp antigens [13] and cytokine production, such as IFN-γ, TNF-α and IL-12 [14–17]. These cytokines are required for macrophage activation and death of intracellular parasites [14-17]. Regulation of the effector function of macrophages and T cells seems to depend on microRNAs (miRNAs), small non-coding RNAs of approximately 21 nucleotides in length that are transcribed in the cell nucleus and function as transcriptional regulators of gene expression, regulating the translation of key proteins for the immune response [18,19]. Recent studies have characterized the miRNA profile in visceral leishmaniasis in vitro [20–23] and in vivo [24,25], but the regulatory role of these miRNAs in the immune response is poorly characterized.

In this study, we demonstrated that miRNAs are differentially expressed in splenic leukocytes (SL) of dogs with CVL compared to control dogs. The miR 21 regulatation of IL-12 production and polarization of the immune response by T2 modifying the T1/T2 profile, contributes to increase of the parasite load.

2. MATERIAL AND METHODS

2.1. Dog screening and sample collection

This study was approved by the Committee for Ethics in Animal Experimental Research (COBEA), with the approval of the Committee for Ethics in Animal Use (CEUA) of São Paulo State University (UNESP), School of Veterinary Medicine, Araçatuba according to process 00978/2016.

Two-year-old male and female dogs of both sexes were selected from the Araçatuba Zoonoses Control Center or from owners who signed a free and informed consent form.

For the microarray, four dogs were used as controls, and upon clinical examination, complete blood count (S1a Table) and serum biochemical profile, were within normal rabger for the species (S1b and S1c Table), and were also negative (serological and molecular diagnosis) for leishmaniasis (S2 Table), and eight dogs naturally infected by *L. infantum* [26] with at least three clinical signs characteristic of the disease, including onychogrifosis, weight loss, ear-tip injuries, periocular lesions, alopecia, skin lesions and lymphadenopathy and with serological and molecular diagnosis positive for leishmaniasis (Table S3). Infected dogs were classified in the clinical stage II of the disease based on the results of the blood count (S4a Table) and biochemical profile (S4b and S4c Table) [27].

For the transfection with miScript miRNA Mimic and Inhibitor (Qiagen™, USA) in the SL, four control dogs with negative serological and molecular diagnosis for leishmaniasis were used (S5 Table) [25], and eight dogs naturally infected by L. Infantum with positive serological and molecular diagnosis for leishmaniasis (Table S6). The same inclusion criteria described abone were used for the transfection experiment (S7a, S7b and S7c Table).

Five mL of blood from both groups (infected and controls) were collected by jugular vein puncture, 4ml in tubes without EDTA to obtain serum for indirect ELISA for detection of anti-leishmania antibodies [28] and biochemical profile, and 1ml in

tubes with EDTA for hemogram. The infected dogs were euthanized by barbiturate anesthesia (Tiopental, Cristália Itapira, SP), followed by intravenous injection of 19.1% potassium chloride by the same route, in compliance with local legislation. After euthanasia, a $2cm^3$ fragment of the spleen was collected for isolation of splenic leukocytes. Spleen fragments in control dogs were removed by surgical excision [29].

2.2. Isolation of splenic leukocytes

Total spleen leukocytes were obtained from a $2cm^3$ fragment that was macerated and added to 10ml RPMI-1640 medium (Sigma®, USA) supplemented with 10% heat inactivated fetal bovine serum, 0.03% L- glutamine, and 100IU/mL penicillin and 100mg/mL streptomycin. After removal of the cell debris through a 100µm filter (BD Falcon ™ Cell strainer, USA), the suspension was processed with 5mL of red blood cell lysis buffer containing 7.46g/L ammonium chloride (NH4 ClO3) at 4 for 10 minutes, centrifuged at 2000rpm for 5 minutes and washed with phosphate buffered saline at pH 7.2 three times. After obtaining the cells and counting in the Neubauer chamber, DNA and total RNA were extracted.

2.3. Molecular diagnosis by real-time PCR

Parasite load quantification was developed by Real-Time PCR with a final reaction volume of 20µL using primers amplifying a 116bp fragment from the kinetoplast DNA kDNA of Leishmania spp. (5 'CCTATTTTACACCAACCCCCAGT 3' and 5 'GGGTAGGGGCGTTCTGCGAAA 3'), at a concentration of 900nM [30], Power SYBR Green PCR Master Mix (Applied Biosystems, USA) and 50ng of DNA amplification condition used was an initial heating of 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 65°C for 60 seconds. Following amplification, a dissociation curve of the amplified fragment was determined from 60°C to 95°C with an increase of 0.5°C every 5 seconds. For each reaction a standard curve with serial dilution of DNA from *Leishmania infantum* promastigotes (MHOM / BR00 / MER02) was performed.

2.4. Extraction and quantification of total RNA

Extraction of total RNA from splenic leukocytes was performed immediately after sample processing, with the commercial mirVana® kit for isolation of total RNA with phenol (Life Technologies, USA) following the procedure indicated by the

manufacturer. After isolation of the fraction containing to be RNAs, sample were stored at -80°C for determination of quality and concentration.

Isolated RNAs were analyzed by spectrophotometer (NanoDrop, Thermo Scientific, USA) for purity evaluation (260/280) and quantified in fluorimeter (Qubit 3.0, Invitrogen), following the manufacturer's instructions. Before performing microarray, sample, were also analyzed for RNA quality by capillary electrophoresis (Bioanalizer, Agilent Technologies, USA) using the commercial Agilent RNA 6000 Nano Kit following the manufacturer's instructions.

2.5. Microarray

Total RNA (250ng)with aRIN above 8 were used to perform the microarray using the miRNA 4.1 Array Strip (Affymetrix, USA) containing probes designed for miRNAs of different species.

miRNAs were biotinylated using the Affymetrix FlashTag™ Biotin HSR RNA Labeling Kit commercial kit following the manufacturer's instructions. For the microarray, GeneAtlas® Hybridization, Wash, and Stain Kit for miRNA array Strips were used, following the manufacturer's instructions.

The microarray data were deposited in the Gene Expression Omnibus database with accession number GSE112459 according to minimum information about microarray experiment (MIAME) standards.

2.6. Microarray data analysis

The normalization and quality of microarray analysis of miRNAs of control and infected dogs were performed in the Expression Console Software, version 1.4.1 program. (Affymetrix, Thermo Fisher Scientific, USA). Differential analysis of the miRNAs was performed in the Transcriptome Analysis Console (Affymetrix, Thermo Fisher Scientific, USA).

Targets and pathways of differentially expressed miRNAs in dogs with VL were analyzed using the Ingenuity Pathway Analysis program (Qiagen®, USA).

Enrichment analysis of Gene Ontology (GO) terms was performed using the ENRICHR program (http://amp.pharm.mssm.edu/Enrichr/) [31,32].

2.7. Real-time PCR for miRNAs validation

Microarray were validated results by real-time PCR (qPCR). CDNA production was performed using the miScript RT II kit (Qiagen™) as recommended by the manufacturer. Reactions of qPCR were performed using commercially available specific primers for the *Canis familiaris* miRNAs and the endogenous reference SNORD96A (miScript, Qiagen™) using the SYBR Green system (myScript SYBR Green PCR kit, Qiagen™) in a real-time thermal cycler (RealPlex, Eppendorf™). Amplification conditions were determined by the manufacturer. For each miRNA, a standard curve was performed with serial dilution of a pool of cDNAs.. Absolute quantification of each miRNA was performed by converting the sample cycle threshold values to a concentration (ng/µl), based on the standard curves, which were generated using serial 10-fold serial dilutions of the cDNA pool. The target amount was then divided by the SNORD96A levels to obtain a normalized target value. All samples were evaluated in duplicate.

2.8. Transfection to increase or decrease miR 21 in splenic leukocytes

Splenic leukocytes were cultured (10^4 cells/replicate) in triplicate in a 96-well plate for 67h at 37°C in the 5% CO2. Negative control of mimic, miR 21 mimic, miR 21 inhibitor and transfection control (miScript miRNA Mimic and Inhibitor Qiagen™, USA) were used at a final concentration of 75nM and using 1.5µL of Hiperfect (Qiagen™, USA) by replica, following manufacturer's instructions. For the evaluation of the transfection rate the AllStars Hs Cell Death Control siRNA (Qiagen™, USA) was used following manufacturer's guidelines.

2.9. Dosage of IL-12

After transfection, SL were incubated for 67h at 37°C in the 5% CO2 and supernatants were collected, centrifuged at 2500rpm and stocked at -80°C. Concentration of IL-12 in the supernatant was determined by capture ELISA using

Canine IL-12/IL-23 p40 DuoSet ELISA Kit (R&D Systems, USA) according to the manufacturer's guidelines.

2.10. Quantification of parasite load in the cell culture by flow cytometry

After transfection, SL were incubated for 67 h at 37°C in the 5% CO2 and parasite load determined by flow cytometry. The method described by [33] was used, with modifications. SL (10^4 cells / treatment) were fixed with 1% paraformaldehyde for 60 min at room temperature and permeabilized in ethanol for 60 min at -20°C. SL were incubated with GP63 monoclonal antibody (ABD, Serotec, USA) at 4°C for 60 min. After three successive washes with phosphate buffered saline at pH 7.2 with 2% BSA (bovine serum albumin), they were stained with goat anti-mouse IgG2a conjugated to phycoerythrin (PE) (R&D Systems, USA) and monoclonal antibody anti-human CD14 conjugated fluorescein isothiocyanate (FITC) (Bio Rad, USA) for 60 minutes at 4°C. After incubation with the secondary antibody, the SLs were washed in phosphate buffered saline at pH 7.2 with 2% BSA, and resuspended in phosphate buffered saline at pH 7.2. Flow cytometry was performed on the Accuri C6 cytometer (BD Biosciences, USA). Acquisition of 10,000 events were counted by experimental replica on channel FL1 and FL2 and cytometric analysis was performed with an Acuri C5 flow cytometer (BD Biosciences, USA) using BD Accuri C6 software, version 1.0.264.21 (BD Biosciences, CA, USA).

2.11. Flow cytometric analysis for labeling of transcription factors T-bet (T1) and GATA 3 (T2) in splenic leukocytes

For staining of T-bet and GATA-3 the SL were fixed and permeabilized with commercial buffer (eBioscience Bioscience, USA) according to manufacturer's instructions. Cells were incubated with FITC conjugated anti-human monoclonal antibody (R&D Systems, USA) and with PE conjugated anti-human monoclonal GATA3 (R & D Systems, USA), and control isotypes (R&D Systems, USA), following manufacturer's instructions. According to a previous study that assessed the similarities between the T-bet and GATA3 protein sequences of Homo sapiens and Canis lupus familiaris using BLAST (basic local alignment search tool) algorithm of the National Center for Biotechnology Information (NCBI), human T-bet and GATA3

showed, respectively, 93% and 97% homology with the canine protein. Acquisition of 10,000 events were counted by experimental replica on channel FL1 and FL2 and cytometric analysis was performed with an Acuri C5 Flow Cytometer (BD Biosciences, USA) using BD Accuri C6 software, version 1.0.264.21 (BD Biosciences, CA, USA).

Mean fluorescence obtained on T-bet positive cells was divided by the mean fluorescence of positive GATA 3 cells to create a T-Bet (Th1)/GATA 3 (Th2) ratio for each treatment.

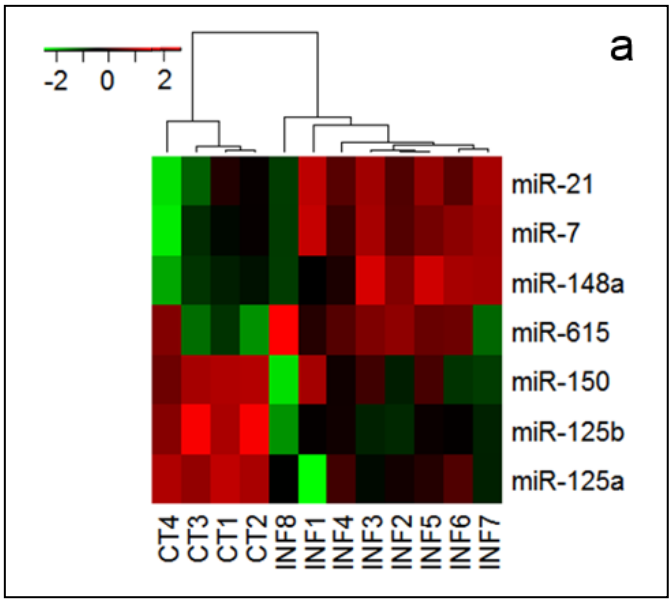
2.12. Statistical analysis

Statistical analyzes were performed using the GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). For the comparison of groups in the microarray, analysis of variance (ANOVA) was used. Real-time PCR values for miRNAs, were tested by Mann-Whitney test correlation between T-bet and GATA-3, the IL-12 dosage and the parasite load dosage was done by Friedman Test with multiple comparison. Results were considered significant when $p < 0.05$.

3. RESULTS

3.1. miRNAs differentially expressed in SL of dogs with LVC compared to control dogs.

Given that immunological response can be regulated by the miRNAs [18], we performed microarray for comparative analysis of expression of the miRNAs in SL of dogs naturally infected by *L. infantum* and control dogs. We observed that miR 21, miR 148a, miR 7 and miR 615 miRNAs showed increased expression (3.2, 2.3, 2.4 and 2.3 times, respectively), while miRNAs miR150, miR125a, miR125b decreased expression in LVC. (Fig. 1a,(Fig. 1b). Canine miRNAs hybridized also to the microarray with probes of other species (mammals, birds, fish, plants), but none of them showed homology with the currently available canine sequences currently known, Percentage of canine miRNAs increased was 1.37% (4/291) and decreased by 1.03% (3/291). To confirm the microarray results, real-time PCR validation was performed. Reactions showed efficiency of 0.98 to 1.13 and R^2 of 0.947 to 0.994(S8 Table). miRNAs miR 148a, miR 615 and miR 21 increased in the SL by qPCR similarly to microarray results, as shown in figure 1c.


a
-2
0
2
miR-21
miR-7
miR-148a
miR-615
miR-150
miR-125b
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CT4
CT3
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INF8
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INF4
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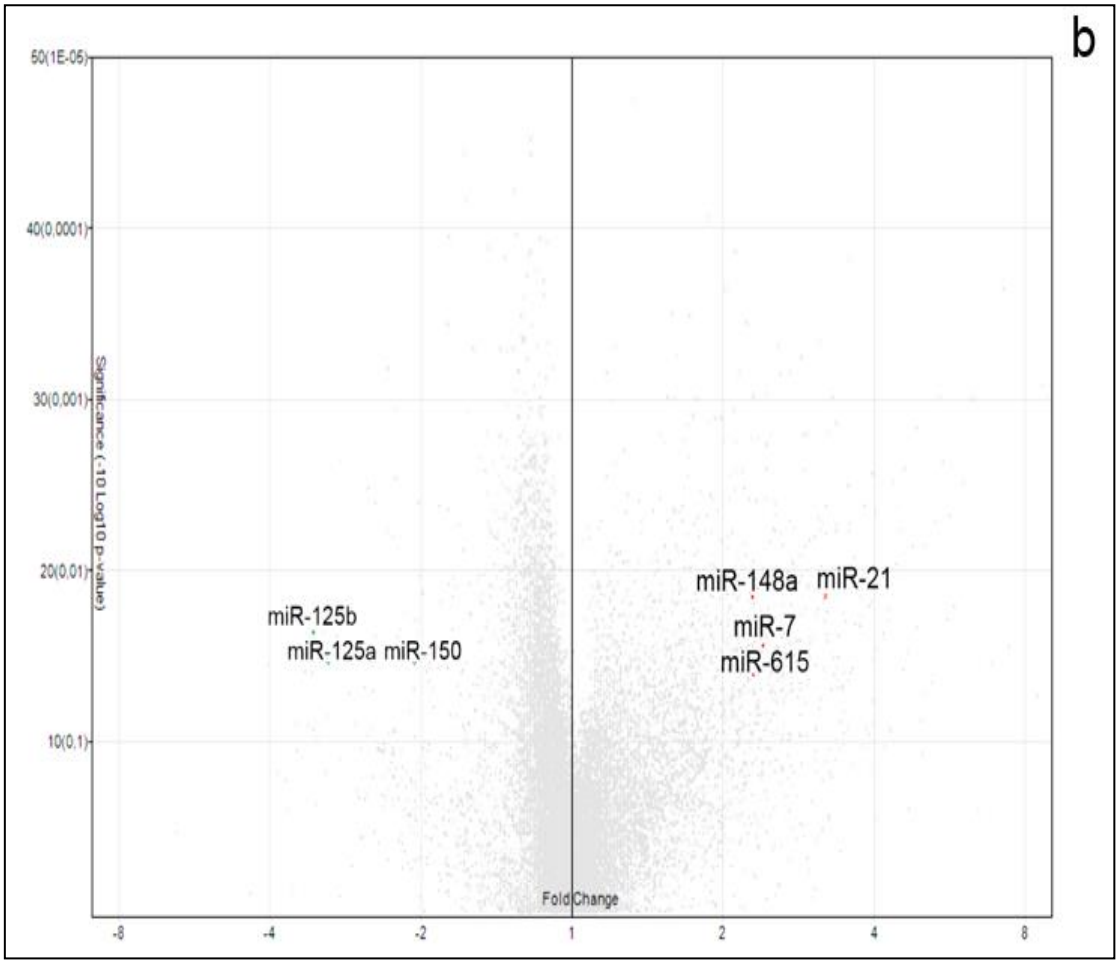
INF6
INF7


b
50(1E-05)
40(0.0001)
30(0.001)
20(0.01)
10(0.1)
Significance (-10 Log10 p-value)
miR-125b
miR-125a miR-150
miR-148a
miR-21
miR-7
miR-615
Fold Change
-8
-4
-2
1
2
4
8

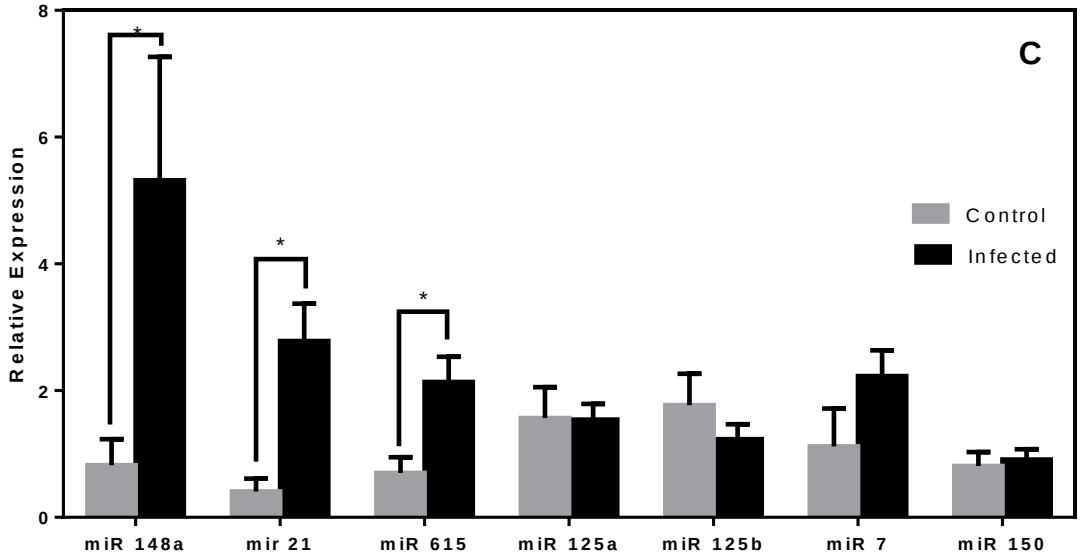


FIGURE 1: Differentially expressed miRNAs in SL of dogs with VL. Representation of the 7 miRNAs differentially expressed in LE of dogs with VL in comparison to healthy dogs. (a) Heat map lines represent individual miRNAs and the columns represent independent replications (eight dogs with VL and four control dogs). The color scale represents normalized expression levels of miRNAs in the two conditions on the log2 scale; red denotes up-regulation and green denotes down-regulation, respectively. Fold Change was less than -2 and greater than 2. (b) Vulcan. On the left are miRNAs with negative expression (green) and miRNAs with positive expression (red). Analysis of variances (ANOVA) was used between groups, and the result was considered significant when $p < 0.05$. (c) Expression of the miRNAs in the infected and control groups was quantified by real-time PCR in the SL. The data represent the mean values of the miRNA expression + standard error of the mean and the asterisks represent the statistically significant data of the Mann-Whitney test.

3.2. Canonical pathways and target genes regulated by miRNAs differentially expressed in the spleen of infected animals

IPA target filter (highly predicted and observed experimentally) showed and demonstrated 114 canonical pathways regulated by these miRNAs and gene targets (Table 9). Top 30 canonical pathways and target genes are shown in Table 1, including p53 signaling, PTEN signaling, STAT3 pathway, death receptor and crosstalk signaling between dendritic cells, p38 MAPK signaling, and activation of Th1/Th2 pathways that can regulate the immune response in CVL.

Table 1. Thirty major canonical pathways predicted for miRNAs differentially regulated in CVL.

Ingenuity Canonical Pathways	P value	Target miRNAs	Target genes
Neuregulin Signaling	0,000144544	miR 148a	CDK5R1, ERBB3, ERRFI1, MRAS, NRAS, PRKCZ, PTEN, SOS1, SOS2, TGFA
		miR 21	PIK3R1, PTEN
		miR 615	PRKCG
STAT3 Pathway	0,001	miR 148a	FLT1, MAP3K9, MRAS, NRAS, TGFA
		miR 21	BMPR2, CDC25A, CDKN1A, SOCS5, TGFBR2
		miR 615	MAPK13
PTEN Signaling	0,001	miR 148a	FLT1, MRAS, NRAS, PRKCZ, PTEN, SOS1, SOS2
		miR 21	BMPR2, CDKN1A, FASLG, PIK3R1, PTEN, TGFBR2
HER-2 Signaling in Breast Cancer	0,001548817	miR 148a	ERBB3, MRAS, NRAS, PRKCZ, SOS1, SOS2
		miR 21	CDK6, CDKN1A, PIK3R1
		miR 615	PRKCG
Myc Mediated Apoptosis Signaling	0,001548817	miR 148a	MRAS, NRAS, PRKCZ, SOS1, SOS2
		miR 21	APAF1, FAS, FASLG, PIK3R1
Glioma Signaling	0,001862087	miR 148a	MRAS, NRAS, PRKCZ, PTEN, SOS1, SOS2, TGFA
		miR 21	CDK6, CDKN1A, PIK3R1, PTEN
		miR 615	PRKCG
Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells	0,001905461	miR 148a	HLA-A, HLA-B, HLA-C
		miR 21	APAF1, FAS, FASLG
Neurotrophin/TRK Signaling	0,001905461	miR 148a	MRAS, NRAS, SOS1, SOS2
		miR 21	NTF3, PIK3R1, SPRY1, SPRY2
		miR 615	NTF4

ErbB Signaling	0,001905461	miR 148a	ERBB3, MRAS, NRAS, PRKCZ, SOS1, SOS2, TGFA
		miR 21	PIK3R1, MAPK13
		miR 615	PRKCG
Cholecystokinin/Gastrin-mediated Signaling	0,002187762	miR 148a	CCKBR, FLT1, MRAS, NRAS, PRKCZ, ROCK1, SOS1, SOS2
		miR 21	ACTA2, EIF1AX, PIK3R1, TNF
		miR 615	MEF2A, PRKCG
Type I Diabetes Mellitus Signaling	0,002691535	miR 148a	HLA-A, HLA-B, HLA-C
		miR 21	APAF1, FAS, FASLG, IL12A, SOCS5, TNF
		miR 615	MAPK13
TGF-β Signaling	0,003235937	miR 148a	INHBB, MRAS, NRAS, SOS1, SOS2
		miR 21	BMPR2, SMAD7, TGFBR2
		miR 615	MAPK13
VEGF Family Ligand-Receptor Interactions	0,003235937	miR 148a	FLT1, MRAS, NRAS, NRP1, PRKCZ, SOS1, SOS2
		miR 21	PIK3R1
		miR 615	PRKCG
Molecular Mechanisms of Cancer	0,003890451	miR 148a	CDK19, MRAS, NRAS, PRKCZ, SOS1, SOS2
		miR 21	APAF1, BMPR2, CDC25A, CDK6, CDKN1A, FAS, FASLG, PIK3R1, SMAD7, TGFBR2
		miR 615	MAPK13, MAX, PRKCG, RALBP1
Th2 Pathway	0,005623413	miR 148a	BHLHE41, HLA-A, HLA-B, HLA-DQB2, MAF, S1PR1
		miR 21	BMPR2, IL12A, JAG1, PIK3R1, TGFBR2
VEGF Signaling	0,007079458	miR 148a	FLT1, MRAS, NRAS, ROCK1, SOS1, SOS2
		miR 21	ACTA2, EIF1AX, PIK3R1
Th1 and Th2 Activation Pathway	0,007079458	miR 148a	BHLHE41, HLA-A, HLA-B, HLA-DQB2, MAF, S1PR1
		miR 21	BMPR2, IL12A, IL6R, JAG1, PIK3R1, TGFBR2
Graft-versus-Host Disease Signaling	0,007079458	miR 148a	HLA-A, HLA-B, HLA-C
		miR 21	FAS, FASLG, TNF
p53 Signaling	0,007079458	miR 148a	GADD45A, JMY, MDM4, PTEN

		miR 21	APAF1, CDKN1A, FAS, PIK3R1, PTEN, SERPINB5
Prolactin Signaling	0,007079458	miR 148a	MRAS, NRAS, PRKCZ, SOS1, SOS2
		miR 21	PIK3R1, SOCS5
		miR 615	PRKCG
Crosstalk between Dendritic Cells and Natural Killer Cells	0,007079458	miR 148a	HLA-A, HLA-B, HLA-C
		miR 21	ACTA2, FAS, FASLG, IL12A, TNF
JAK/Stat Signaling	0,007079458	miR 148a	CCKBR, MRAS, NRAS, SOS1, SOS2
		miR 21	CDKN1A, PIK3R1, SOCS5
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	0,007762471	miR 148a	GADD45A, MDM4, PRKCZ, SKP1
		miR 21	CDKN1A, SKP2
Virus Entry via Endocytic Pathways	0,00851138	miR 148a	HLA-A, HLA-B, HLA-C, MRAS, NRAS, PRKCZ
		miR 21	ACTA2, PIK3R1
		miR 615	PRKCG
HGF Signaling	0,01	miR 148a	MAP3K9, MRAS, NRAS, PRKCZ, SOS1, SOS2
		miR 21	CDKN1A, PIK3R1
		miR 615	PRKCG
p38 MAPK Signaling	0,01023293	miR 148a	RPS6KA5
		miR 21	FAS, FASLG, TGFBR2, TNF
		miR 615	HSPB7, MAPK13, MAX, MEF2A
ErbB2-ErbB3 Signaling	0,010471285	miR 148a	ERBB3, MRAS, NRAS, PTEN, SOS1, SOS2
		miR 21	PIK3R1, PTEN
Antigen Presentation Pathway	0,011220185	miR148a	HLA-A, HLA-B, HLA-C, HLA-DQB2, PDIA3
Fc Epsilon RI Signaling	0,011220185	miR 148a	MRAS, NRAS, PRKCZ, SOS1, SOS2
		miR 21	PIK3R1, TNF
		miR 615	MAPK13, PRKCG
NGF Signaling	0,011220185	miR 148a	MAP3K9, MRAS, NRAS, PRKCZ, ROCK1, RPS6KA5, SOS1, SOS2
		miR 21	PIK3R1

To understend the functional networks of the target genes, GO terms were analyzed. Analysis showed that these target genes were involved in a large number of

biological processes where from a total of 1,101 entries, 30 are presented in Table S10afor cellular component, a total of 74 entries, 30 are presented inTable S10b, molecular function, a total of 151 entries were fowed, and 30 are presented in Table S10c.

3.3. MiR-21 plays an important role in the inhibition of IL-12

As IL-12 is an important cytokine for activation of NK and INF-γ production by T cell [33], polarization of immune response to T1 [34] and is induced during CVL infection [35], we investigated its potential involvement with miR 21 using miScript miRNA Mimic and Inhibitor (Qiagen™, USA). We observed an increase in IL-12 increased in the supernatants of SL cultures of dogs naturally infected by *L. infantum*, following inhibition of miR-21 (Fig. 2a). In control dogs, SL levels of IL-12 decreased in the culture supernatants, but not statistical significanted, possibly due to low number on dogs used and low expression of miR 21 (Fig 2b).

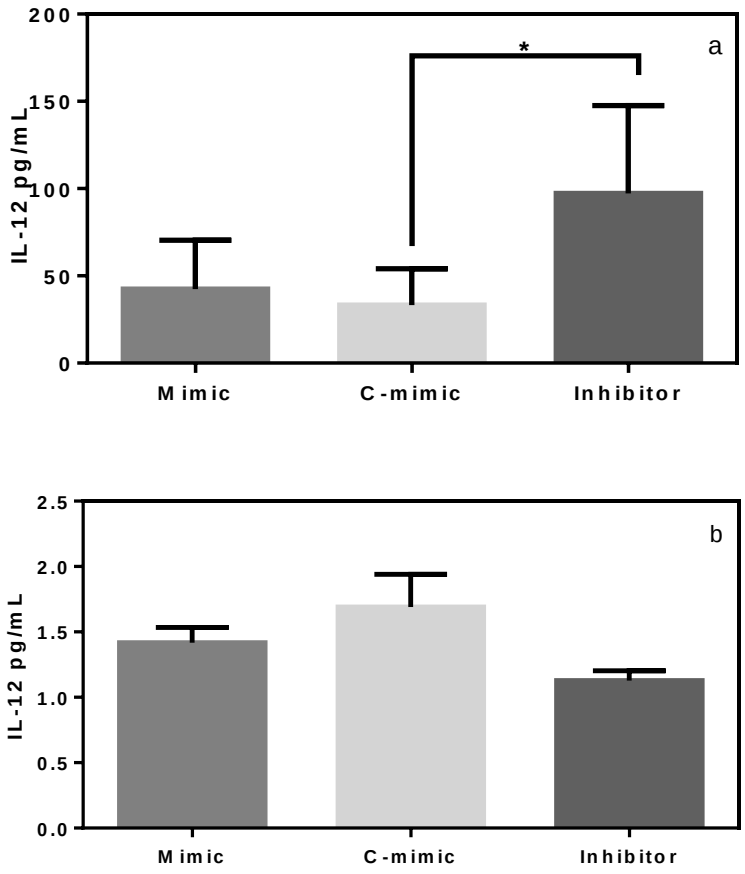


FIGURE 2: IL-12 production was quantified in supernatants from SL cultures of dogs transfected with negative control of mimic, miR 21 mimic, miR 21 inhibitor, all in the presence of Hiperfect (miScript miRNA Mimic and Inhibitor Qiagen™, USA), following for 67h

in culture. (a) LE from dogs naturally infected by L. infantum and (b) SL from control dogs. Data represent the mean values of the IL-12 production + SE Asterisks represent statistical significant on the Friedman Test with multiple comparison.

3.4. Inhibition of miR-21 increases T1 response

To assess whether miR-21 in playing a role in the polarization of immune response to T1 or T2 in dogs infected by *L. infantum*, after transfection of SL to increase or inhibit miR 21, T-bet and GATA 3 transcription factors were evaluated signaling T1 and T2, respectively [36]. Inhibition of miR21 increased T1 profile population in the SL of dogs with VL (Fig. 3a), whereas in the SL of control dogs miR 21 mimic showed a tendency of decrease but not statistically significant, possibly due to low number and low concentration of mimic miR 21 (Fig. 3b). Representative images of the histogram of cytometry are demonstrated in S11 Figure.

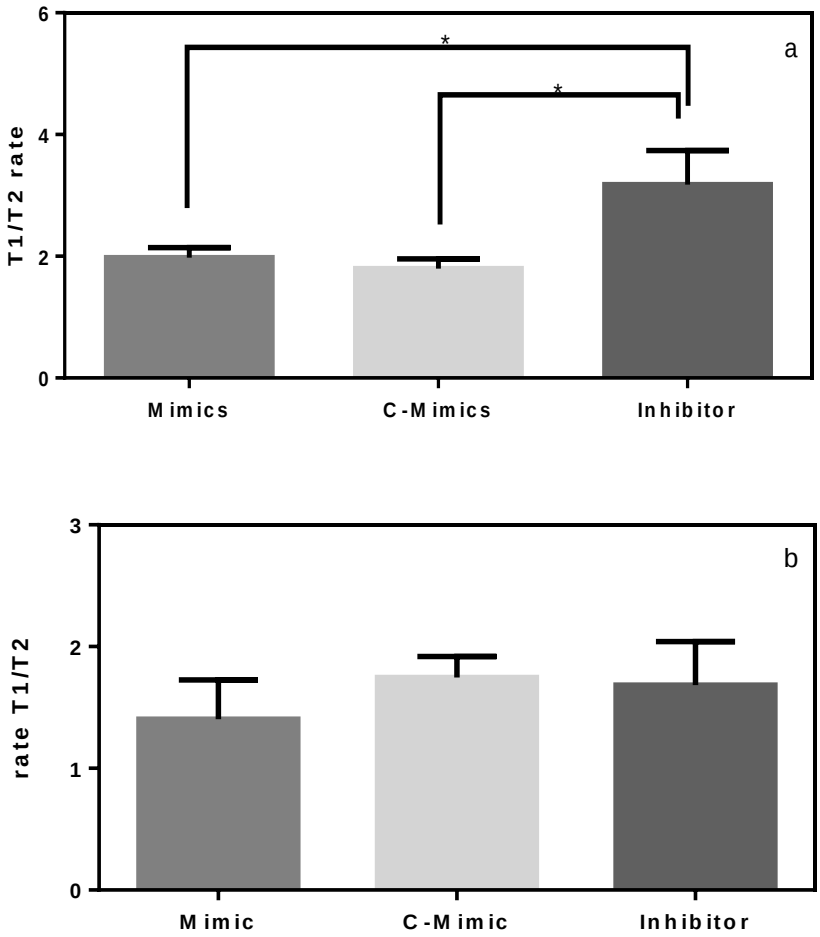


FIGURE 3: T1 profile in cultures of the SLs transfected with negative control of mimic, miR 21 mimic, miR 21 inhibitorand cultured for 67h. (a) SL from dogs naturally infected

by L. infantum and (b) SL from control dogs. The data represent the mean values of the ratio T1/T2 + standard error of the mean and the asterisks represent the statistically significant data through the Friedman test with multiple comparison.

3.5. Inhibition of miR 21 decreases parasitic load

Parasitic load is important in the progression of CVL [37]. To assess whether miR-21 was related to its control, decrease of miR 21 in the SL by transfection with miR 21 inhibitor, or increase of miR21 in the SL by transfection of miR 21 mimics was performed. We observed with the inhibition of miR 21 was following by a decrease of parasitic load in SL of dogs with VL. (Fig. 4). Representative images of the histogram of cytometry are demonstrated in S12 Figure.

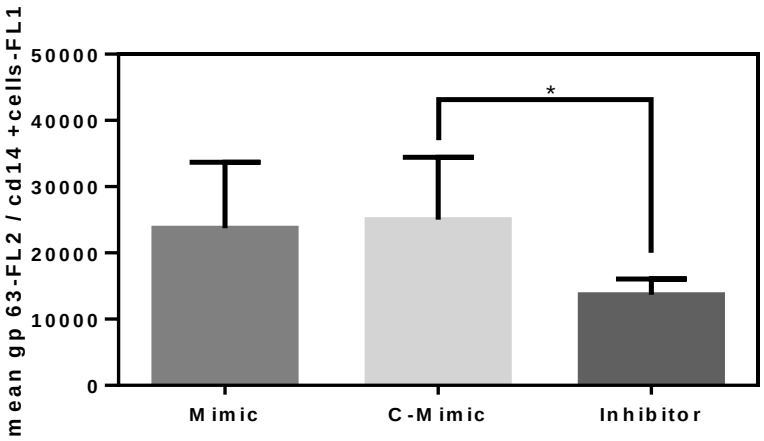


FIGURE 4: Parasitic loading in cultures of naturally infected L. infantum and transfected with negative control of mimic, miR 21 mimic, miR 21 inhibitorand cultured for 67h. The data represent the values of the mean + standard error of the mean and the asterisks represent the statistically significant data through the Friedman test with multiple comparison.

4. DISCUSSION

We demonstrated by microarray seven miRNAs differentially expressed in SL of dogs with LVC in comparison with control animals; the miRNAs: miR 21, miR 148a, miR 7 and miR 615 showed increased expression, and the miRNAs: miR 150, miR 125a and miR 125b showed decrease in expression. The miRNAs differentially expressed in the microarray in the *Canis familiaris* species were also evaluated by real-time PCR, confirming increased miRNAs: miR 21, miR 148a and miR 615. miR 21 was

transfected with mimic and inhibited tools in the SL of dogs with CVL and modulating IL-12 cytokine levels, regulating the polarization of the immune response, to the T1 profile and regulating the parasite load.

MicroRNAs diferencially expressed in the LS of dogs with LVC differed from previas studies, Differential expression of miR 122 was demonstrated in mice macrophages infected by L. infantum [38], In human macrophages infected by *L. donovani* members of the miR 30A-3p family were observed [21] suggesting that it is indispensable to study the dog because it is the most important reservoir of the disease.

miR 148a showed a significant increase of 2.29 times in SL of dogs with VL. An exogenous increase in the expression of this miRNA may induce apoptosis in colon cancer cells by silencing Bcl-2 [39]. In dogs with VL high levels of apoptosis of CD4 + and CD8 + cells are observed in both blood and spleen, when compared to healthy animals [40]. miR 148a targets important genes for the regulation of apoptosis such as FAS and FASLG, suggesting in the death of CD4 + and CD8 + cells in VL dogs.

miR 615 showed increased expression of 2.3 fold in LE of dogs with VL. This miRNA targets ligand-dependent nuclear corepressor (LCoR), a derepressor of peroxisome proliferator-activated receptor gamma (PPARγ), which promotes the phagocytic capacity of splenic macrophages in mice [41]. In the spleen of dogs with VL, there is an increase in proliferation and differentiation of macrophages [42,43]. Therefore, an increase in the expression of this miRNA may increase phagocytic capacity of macrophage in an attempt to combat infection by *L. infantum*.

miR 21 presented a significant increase (3.7 fold) in SL of dogs with VL. This miRNA is involved with immune regulation as a negative modulator of T cell activation in human [44], and a negative correlation was observed witer TNF-alpha and IL-6 production in human PBMC [45], In addition, the presence of TNF-α is associated with resistance to canine visceral leishmaniasis [46], thus, high levels of miR 21 may be decreasing TNF-α, which could increase splenic parasitic burden, contributing to disease progression.

Targets of the differentially expressed miR148a, miR615 and miR21 in SL from *L. infantum* infected dogs were explored to canonical pathways, and immunity-related pathways were identified such as STAT3, PTEN, cytotoxic T cells mediating apoptosis TGF-β, Th2 activation, Th1 and Th2 activation, p53, Cross Talk between dendritic cells and NK, JAK/Stat, p38 MAPK, and antigen presentation, amongst

others. Some genes from these pathways have already been described in the disease. In humans with visceral leishmaniasis, PTEN is actively involved in susceptibility to the disease, its decrease in splenic tissue correlates with an increase in IL-10 cytokine [47]and similar results were observed in macrophages of mice with *L. major* [48]. Infection of macrophages from mice with *L. donovani* increases signaling of p38MAPK [49]. *In vitro* infection of human phagocytes with *L. donovani* showed that differentially expressed miRNAs interfere with JAK-STAT and TGF-β signaling pathways [20]. Therefore, these pathways may be important in directing future studies on immune responses in visceral leishmaniasis.

Transfection of miR21 was carried out due to its involvement in immune response regulation [49]. Inhibition of mirR 21 in SL from dogs with VL showed that miR21 targets IL-12 cytokine, confirming the involvement of miR 21 in IL-12 expression in the canine model Similar results were observed in mouse dendritic cells following LPS stimulation, where miR 21 deficiency increased IL-12 production [50]. miR 21 binds to the 3 'untranslated region of IL-12p35, which is conserved in several species [51], regulating the production of IL-12. IL-12 is a heterodimeric cytokine produced by most inflammatory cells in response to intracellular pathogens. IL-12 induces the production of IFN-ɣ by NK cells and T cells, and is important for the maintenance of Th1-type response [52]. IL-12 neutralization causes amastigote replication in the spleen of BALB / c mice, and the IL-12 knockout mouse has greater parasite burden on the liver and spleen than control [53–55]. In LVC, IL-12 has been observed in the spleen [56–58], but the amount produced seems not to be sufficient to maintain a strong Th1-type response, since exogenous IL-12 increases PBMC from dogs proliferation, and IFN-γ production [56]. High production of miR 21 in naturally infected dogs decreases IL-12 production, possibly favoring parasite replication in the spleen.

Another effect observed by inhibition of miR 21 was the increase in the T-bet / GATA 3 ratio in SL of dogs with leishmaniasis, confirming the role of miR 21 in the polarization of the T1 response, as previously observed in a model of allergic inflammation in mice [51]. In dogs, the progression of VL is associated with inhibition of protective T1 response [59,60], and with increased response of non-protective Th2 antibody response [61], thus it is possible that the decrease in miR 21 favored an increase in IL-12, responsible for T1 polarization in naturally infected dogs.

In addition, inhibition of miR 21 also promoted a decrease in parasitic load in SL of infected dogs Decreased parasite burden has been associated with increased

TNF-α, and the asymptomatic condition in dogs seems to depend on differential expression of Th1 cytokines [46]. Increase of miR 21 in mice is associated with the M2 phenotype of macrophages characterized by high production of IL-10, increased arginase 1 and decreased TNF [62]; on the other hand, it has been observed in mice, that the deficiency of miR 21 promotes macrophage polarization for M1 [63]. In dogs with VL, the M2 phenotype predominates among macrophages in the spleen, where high parasitic load is observed [61,64], therefore, inhibition of miR 21 may have increased the percentage of macrophages with profile M1 producing nitric oxide, decreasing load parasitic.

Another possibility for the decrease in parasite load observed with inhibition of miR 21 is related to the activation of T cells. Expression of miR 21 affects ERK phosphorylation and AP-1 activity by inhibiting T cell activation and production of IFN-ɣ [44]. In mice, dysfunction of cellular immunity with VL was attributed to the dephosphorylation of key molecules involved in signaling, leading to inactivation of T cells [65] Inhibition of miR 21 may have promoted the reduction of parasite burden by restoring the signaling involved in T cell activation, further studies are needed to confirm this hypothesis in dogs with leishmaniasis.

Increased production of miR 21, targeting IL-12 protein and impairing immune response, has also been observed in infection by other intracellular pathogens. Increase of miR-21 induced by vaccination with Bacillus Calmette-Guerin (BCG) in mice suppresses IL-12 production, targeting IL-12p35, impairing anti-mycobacterial T cell responses both in vitro and in vivo [66]. Brucella Omp25 infection in human macrophages induces miR-21-5p and negatively regulates IL-12 production at both transcriptional and post-transcriptional, levels impairing macrophage function [67], suggesting that the induction of miR 21 may be involved with an escape mechanism common, to intracellular pathogens of macrophages.

5. CONCLUSION

We conclude that *L. infantum* infection alters expression of miRNAs and that miR 21 interferes in the cellular immune response of *L. infantum*-infected dogs.

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REFERENCES

1. Moreno J, Alvar J. Canine leishmaniasis: Epidemiological risk and the experimental model. Trends Parasitol. 2002;18: 399–405. doi:10.1016/S1471-4922(02)02347-4
2. Murray HW, Berman JD, Davies CR, Saravia NG. Advances in leishmaniasis. Lancet. 2005;366: 1561–1577. doi:10.1016/S0140-6736(05)67629-5
3. Sanches L da C, Martini CC de, Nakamura AA, Santiago MEB, Dolabela de Lima B, Lima VMF de. Natural canine infection by Leishmania infantum and Leishmania amazonensis and their implications for disease control. Rev Bras Parasitol Veterinária. 2016;25: 465–469. doi:10.1590/s1984-29612016071
4. Desjeux P. Leishmaniasis: current situation and new perspectives. Comp Immunol Microbiol Infect Dis. 2004;27: 305–318. doi:10.1016/j.cimid.2004.03.004
5. Mancianti F, Gramiccia M, Gradoni L, Pieri S. Studies on canine leishmaniasis control. 1. Evolution of infection of different clinical forms of canine leishmaniasis following antimonial treatment. Trans R Soc Trop Med Hyg. 1988;82: 566–7. Available: http://www.ncbi.nlm.nih.gov/pubmed/3076714
6. Badaró R, Jones TC, Lorenço R, Cerf BJ, Sampaio D, Carvalho EM, et al. A prospective study of visceral leishmaniasis in an endemic area of Brazil. J Infect Dis. 1986;154: 639–49. Available: http://www.ncbi.nlm.nih.gov/pubmed/3745974
7. Semião-Santos SJ, el Harith A, Ferreira E, Pires CA, Sousa C, Gusmão R. Evora district as a new focus for canine leishmaniasis in Portugal. Parasitol Res. 1995;81: 235–9. Available: http://www.ncbi.nlm.nih.gov/pubmed/7770430
8. Strauss-Ayali D, Baneth G, Jaffe CL. Splenic immune responses during canine visceral leishmaniasis. Vet Res. 2007;38: 547–564. doi:10.1051/vetres:2007015
9. Reis AB, Martins-Filho OA, Teixeira-Carvalho A, Carvalho MG, Mayrink W, França-Silva JC, et al. Parasite density and impaired biochemical/hematological status are associated with severe clinical aspects of canine visceral leishmaniasis. Res Vet Sci. W.B. Saunders; 2006;81: 68–75.

doi:10.1016/J.RVSC.2005.09.011

10. Reis AB, Martins-Filho OA, Teixeira-Carvalho A, Giunchetti RC, Carneiro CM, Mayrink W, et al. Systemic and compartmentalized immune response in canine visceral leishmaniasis. Vet Immunol Immunopathol. 2009;128: 87–95. doi:10.1016/j.vetimm.2008.10.307
11. Maia C, Campino L. Cytokine and Phenotypic Cell Profiles of *Leishmania infantum* Infection in the Dog. J Trop Med. 2012;2012: 1–7. doi:10.1155/2012/541571
12. Agallou M, Pantazi E, Tsiftsaki E, Toubanaki DK, Gaitanaki C, Smirlis D, et al. Induction of protective cellular immune responses against experimental visceral leishmaniasis mediated by dendritic cells pulsed with the N-terminal domain of Leishmania infantum elongation factor-2 and CpG oligodeoxynucleotides. Mol Immunol. 2018;103: 7–20. doi:10.1016/j.molimm.2018.08.004
13. Maasho K, McMahon-Pratt D, Raita J, Raud M, Britton S, Soong L, et al. Evaluation of amastigote reactive cells in human cutaneous leishmaniasis caused by Leishmania aethiopica. Clin Exp Immunol. 2003;132: 316–22. Available: http://www.ncbi.nlm.nih.gov/pubmed/12699423
14. Dikhit MR, Das S, Mahantesh V, Kumar A, Singh AK, Dehury B, et al. The potential HLA Class I-restricted epitopes derived from LeIF and TSA of Leishmania donovani evoke anti-leishmania CD8+ T lymphocyte response. Sci Rep. 2018;8: 14175. doi:10.1038/s41598-018-32040-x
15. Best I, Privat-Maldonado A, Cruz M, Zimic M, Bras-Gonçalves R, Lemesre J-L, et al. IFN-γ Response Is Associated to Time Exposure Among Asymptomatic Immune Responders That Visited American Tegumentary Leishmaniasis Endemic Areas in Peru. Front Cell Infect Microbiol. 2018;8: 289. doi:10.3389/fcimb.2018.00289
16. Schwartz J, Moreno E, Calvo A, Blanco L, Fernández-Rubio C, Sanmartín C, et al. Combination of paromomycin plus human anti-TNF-α antibodies to control the local inflammatory response in BALB/ mice with cutaneous leishmaniasis lesions. J Dermatol Sci. 2018; doi:10.1016/j.jdermsci.2018.07.005
17. Bacellar O, D'oliveira A, Jerônimo S, Carvalho EM. IL-10 and IL-12 are the main regulatory cytokines in visceral leishmaniasis. Cytokine. 2000;12: 1228–31. doi:10.1006/cyto.2000.0694
18. Bartel DP. MicroRNAs: Genomics, Biogenesis, Mechanism, and Function. Cell.

2004. doi:10.1016/S0092-8674(04)00045-5

19. Liu G, Abraham E. MicroRNAs in Immune Response and Macrophage Polarization. Arterioscler Thromb Vasc Biol. 2013;33: 170–177. doi:10.1161/ATVBAHA.112.300068
20. Geraci NS, Tan JC, McDowell MA. Characterization of microRNA expression profiles in Leishmania-infected human phagocytes. Parasite Immunol. NIH Public Access; 2015;37: 43–51. doi:10.1111/pim.12156
21. Singh AK, Pandey RK, Shaha C, Madhubala R. MicroRNA expression profiling of Leishmania donovani-infected host cells uncovers the regulatory role of MIR30A-3p in host autophagy. Autophagy. 2016;12: 1817–1831. doi:10.1080/15548627.2016.1203500
22. Muxel SM, Laranjeira-Silva MF, Zampieri RA, Floeter-Winter LM. Leishmania (Leishmania) amazonensis induces macrophage miR-294 and miR-721 expression and modulates infection by targeting NOS2 and L-arginine metabolism. Sci Rep. Nature Publishing Group; 2017;7: 44141. doi:10.1038/srep44141
23. Bragato JP, Melo LM, Venturin GL, Rebech GT, Garcia LE, Lopes FL, et al. Relationship of peripheral blood mononuclear cells miRNA expression and parasitic load in canine visceral leishmaniasis. Afrin F, editor. PLoS One. 2018;13: e0206876. doi:10.1371/journal.pone.0206876
24. Lemaire J, Mkannez G, Guerfali FZ, Gustin C, Attia H, Sghaier RM, et al. MicroRNA Expression Profile in Human Macrophages in Response to Leishmania major Infection. Valenzuela JG, editor. PLoS Negl Trop Dis. 2013;7: e2478. doi:10.1371/journal.pntd.0002478
25. Tiwari N, Kumar V, Gedda MR, Singh AK, Singh VK, Gannavaram S, et al. Identification and Characterization of miRNAs in Response to Leishmania donovani Infection: Delineation of Their Roles in Macrophage Dysfunction. Front Microbiol. Frontiers Media SA; 2017;8: 314. doi:10.3389/fmicb.2017.00314
26. Bragato JP, Melo LM, Venturin GL, Rebech GT, Garcia LE, Lopes FL, et al. Data on differentially expressed miRNAs in dogs infected with Leishmania infantum. Data Br. 2018;17: 218–225. doi:10.1016/j.dib.2018.01.007
27. Solano-Gallego L, Koutinas A, Miró G, Cardoso L, Pennisi MG, Ferrer L, et al. Directions for the diagnosis, clinical staging, treatment and prevention of canine leishmaniosis. Vet Parasitol. Elsevier; 2009;165: 1–18.

doi:10.1016/J.VETPAR.2009.05.022

28. Lima VMF, Gonçalves ME, Ikeda FA, Luvizotto MCR, Feitosa MM. Anti-leishmania antibodies in cerebrospinal fluid from dogs with visceral leishmaniasis. Braz J Med Biol Res. 2003;36.
29. Lima VMF de, Fattori KR, de Souza F, Eugênio FR, Santos PSP dos, Rozza DB, et al. Apoptosis in T lymphocytes from spleen tissue and peripheral blood of L. (L.) chagasi naturally infected dogs. Vet Parasitol. 2012;184: 147–153. doi:10.1016/j.vetpar.2011.08.024
30. Ranasinghe S, Rogers ME, Hamilton JGC, Bates PA, Maingon RDC. A real-time PCR assay to estimate Leishmania chagasi load in its natural sand fly vector Lutzomyia longipalpis. Trans R Soc Trop Med Hyg. 2008;102: 875–882. doi:10.1016/j.trstmh.2008.04.003
31. Chen EY, Tan CM, Kou Y, Duan Q, Wang Z, Meirelles G, et al. Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. BMC Bioinformatics. 2013;14: 128. doi:10.1186/1471-2105-14-128
32. Kuleshov M V., Jones MR, Rouillard AD, Fernandez NF, Duan Q, Wang Z, et al. Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. Nucleic Acids Res. 2016;44: W90–W97. doi:10.1093/nar/gkw377
33. Di Giorgio C, Ridoux O, Delmas F, Azas N, Gasquet M, Timon-David P. Flow cytometric detection of Leishmania parasites in human monocyte-derived macrophages: application to antileishmanial-drug testing. Antimicrob Agents Chemother. 2000;44: 3074–8. Available: http://www.ncbi.nlm.nih.gov/pubmed/11036025
34. Del Vecchio M, Bajetta E, Canova S, Lotze MT, Wesa A, Parmiani G, et al. Interleukin-12: Biological Properties and Clinical Application. Clin Cancer Res. 2007;13: 4677–4685. doi:10.1158/1078-0432.CCR-07-0776
35. Kropf P, Herath S, Klemenz R, Müller I. Signaling through the T1/ST2 molecule is not necessary for Th2 differentiation but is important for the regulation of type 1 responses in nonhealing Leishmania major infection. Infect Immun. 2003;71: 1961–71. Available: http://www.ncbi.nlm.nih.gov/pubmed/12654814
36. Diaz S, da Fonseca IP, Rodrigues A, Martins C, Cartaxeiro C, Silva MJ, et al. Canine leishmaniosis. Modulation of macrophage/lymphocyte interactions by L. infantum. Vet Parasitol. 2012;189: 137–44. doi:10.1016/j.vetpar.2012.05.004
37. Kanhere A, Hertweck A, Bhatia U, Gökmen MR, Perucha E, Jackson I, et al. T-

bet and GATA3 orchestrate Th1 and Th2 differentiation through lineage-specific targeting of distal regulatory elements. Nat Commun. Nature Publishing Group; 2012;3: 1268. doi:10.1038/ncomms2260

38. Torrecilha RBP, Utsunomiya YT, Bosco AM, Almeida BF, Pereira PP, Narciso LG, et al. Correlations between peripheral parasite load and common clinical and laboratory alterations in dogs with visceral leishmaniasis. Prev Vet Med. 2016;132: 83–87. doi:10.1016/j.prevetmed.2016.08.006
39. Silva SC, Silva DF, Almeida TC, Perasoli FB, da Silva ATP, da Silva GN, et al. Behavior of two Leishmania infantum strains—evaluation of susceptibility to antimonials and expression of microRNAs in experimentally infected J774 macrophages and in BALB/c mice. Parasitol Res. Springer Berlin Heidelberg; 2018;117: 2881–2893. doi:10.1007/s00436-018-5979-3
40. Zhang J, Ying Z, Tang Z, Long L, Li K. MicroRNA-148a Promotes Myogenic Differentiation by Targeting the ROCK1 Gene. J Biol Chem. 2012;287: 21093–21101. doi:10.1074/jbc.M111.330381
41. Silva KLO, Melo LM, Perosso J, Oliveira BB, Santos PSP Dos, Eugênio F de R, et al. CD95 (FAS) and CD178 (FASL) induce the apoptosis of CD4+ and CD8+ cells isolated from the peripheral blood and spleen of dogs naturally infected with Leishmania spp. Vet Parasitol. 2013;197: 470–6. doi:10.1016/j.vetpar.2013.07.012
42. Jiang S, Li C, Olive V, Lykken E, Feng F, Sevilla J, et al. Molecular dissection of the miR-17-92 cluster's critical dual roles in promoting Th1 responses and preventing inducible Treg differentiation. Blood. American Society of Hematology; 2011;118: 5487–97. doi:10.1182/blood-2011-05-355644
43. Alexandre-Pires G, Pais D, Correia M, Pina JAE. Leishmaniosis--a report about the microvascular and cellular architecture of the infected spleen in Canis familiaris. Microsc Res Tech. 2006;69: 227–35. doi:10.1002/jemt.20267
44. Baneth G, Koutinas AF, Solano-Gallego L, Bourdeau P, Ferrer L. Canine leishmaniosis - new concepts and insights on an expanding zoonosis: part one. Trends Parasitol. Elsevier; 2008;24: 324–30. doi:10.1016/j.pt.2008.04.001
45. Carissimi C, Carucci N, Colombo T, Piconese S, Azzalin G, Cipolletta E, et al. miR-21 is a negative modulator of T-cell activation. Biochimie. Elsevier; 2014;107: 319–326. doi:10.1016/J.BIOCHI.2014.09.021
46. Mazloom H, Alizadeh S, Esfahani EN, Razi F, Meshkani R. Decreased

expression of microRNA-21 is associated with increased cytokine production in peripheral blood mononuclear cells (PBMCs) of obese type 2 diabetic and non-diabetic subjects. Mol Cell Biochem. Springer US; 2016;419: 11–17. doi:10.1007/s11010-016-2743-9

47. Alves CF, de Amorim IFG, Moura EP, Ribeiro RR, Alves CF, Michalick MS, et al. Expression of IFN-γ, TNF-α, IL-10 and TGF-β in lymph nodes associates with parasite load and clinical form of disease in dogs naturally infected with Leishmania (Leishmania) chagasi. Vet Immunol Immunopathol. 2009;128: 349–358. doi:10.1016/j.vetimm.2008.11.020
48. Sudarshan M, Singh T, Singh B, Chakravarty J, Sundar S. Suppression of host PTEN gene expression for Leishmania donovani survival in Indian visceral leishmaniasis. Microbes Infect. 2016;18: 369–72. doi:10.1016/j.micinf.2015.12.008
49. Kuroda S, Nishio M, Sasaki T, Horie Y, Kawahara K, Sasaki M, et al. Effective clearance of intracellular Leishmania major in vivo requires Pten in macrophages. Eur J Immunol. 2008;38: 1331–40. doi:10.1002/eji.200737302
50. Liu L, Wang L, Zhao Y, Wang Y, Wang Z, Qiao Z. Testosterone attenuates p38 MAPK pathway during Leishmania donovani infection of macrophages. Parasitol Res. 2006;99: 189–193. doi:10.1007/s00436-006-0168-1
51. Lu TX, Hartner J, Lim E-J, Fabry V, Mingler MK, Cole ET, et al. MicroRNA-21 Limits In Vivo Immune Response-Mediated Activation of the IL-12/IFN- Pathway, Th1 Polarization, and the Severity of Delayed-Type Hypersensitivity. J Immunol. 2011;187: 3362–3373. doi:10.4049/jimmunol.1101235
52. Lu TX, Munitz A, Rothenberg ME. MicroRNA-21 is up-regulated in allergic airway inflammation and regulates IL-12p35 expression. J Immunol. NIH Public Access; 2009;182: 4994–5002. doi:10.4049/jimmunol.0803560
53. Seder RA, Gazzinelli R, Sher A, Paul WE. Interleukin 12 acts directly on CD4+ T cells to enhance priming for interferon gamma production and diminishes interleukin 4 inhibition of such priming. Proc Natl Acad Sci U S A. 1993;90: 10188–92. Available: http://www.ncbi.nlm.nih.gov/pubmed/7901851
54. Engwerda CR, Murphy ML, Cotterell SEJ, Smelt SC, Kaye PM. Neutralization of IL-12 demonstrates the existence of discrete organ-specific phases in the control ofLeishmania donovani. Eur J Immunol. 1998;28: 669–680.

doi:10.1002/(SICI)1521-4141(199802)28:02<669::AID-IMMU669>3.0.CO;2-N

55. Murray HW, Montelibano C, Peterson R, Sypek JP. Interleukin-12 Regulates the Response to Chemotherapy in Experimental Visceral Leishmaniasis. J Infect Dis. 2000;182: 1497–1502. doi:10.1086/315890
56. Satoskar AR, Rodig S, Telford SR, Satoskar AA, Ghosh SK, von Lichtenberg F, et al. IL-12 gene-deficient C57BL/6 mice are susceptible to Leishmania donovani but have diminished hepatic immunopathology. Eur J Immunol. 2000;30: 834–9. doi:10.1002/1521-4141(200003)30:3<834::AID-IMMU834>3.0.CO;2-9
57. Strauss-Ayali D, Baneth G, Shor S, Okano F, Jaffe CL. Interleukin-12 augments a Th1-type immune response manifested as lymphocyte proliferation and interferon gamma production in Leishmania infantum-infected dogs. Int J Parasitol. 2005;35: 63–73. doi:10.1016/j.ijpara.2004.10.015
58. Santos-Gomes GM, Rosa R, Leandro C, Cortes S, Romão P, Silveira H. Cytokine expression during the outcome of canine experimental infection by Leishmania infantum. Vet Immunol Immunopathol. 2002;88: 21–30. Available: http://www.ncbi.nlm.nih.gov/pubmed/12088641
59. Lage RS, Oliveira GC, Busek SU, Guerra LL, Giunchetti RC, Corrêa-Oliveira R, et al. Analysis of the cytokine profile in spleen cells from dogs naturally infected by Leishmania chagasi. Vet Immunol Immunopathol. 2007;115: 135–145. doi:10.1016/j.vetimm.2006.10.001
60. Pinelli E, Killick-Kendrick R, Wagenaar J, Bernadina W, del Real G, Ruitenberg J. Cellular and humoral immune responses in dogs experimentally and naturally infected with Leishmania infantum. Infect Immun. 1994;62: 229–35. Available: http://www.ncbi.nlm.nih.gov/pubmed/8262632
61. Pinelli E, Gonzalo RM, Boog CJ, Rutten VP, Gebhard D, del Real G, et al. Leishmania infantum-specific T cell lines derived from asymptomatic dogs that lyse infected macrophages in a major histocompatibility complex-restricted manner. Eur J Immunol. 1995;25: 1594–600. doi:10.1002/eji.1830250619
62. Koutinas AF, Koutinas CK. Pathologic Mechanisms Underlying the Clinical Findings in Canine Leishmaniosis due to *Leishmania infantum/chagasi*. Vet Pathol. 2014;51: 527–538. doi:10.1177/0300985814521248
63. Sheedy FJ. Turning 21: Induction of miR-21 as a Key Switch in the Inflammatory Response. Front Immunol. 2015;6. doi:10.3389/fimmu.2015.00019
64. Xi J, Huang Q, Wang L, Ma X, Deng Q, Kumar M, et al. miR-21 depletion in

macrophages promotes tumoricidal polarization and enhances PD-1 immunotherapy. Oncogene. NIH Public Access; 2018;37: 3151–3165. doi:10.1038/s41388-018-0178-3

65. Moreira PRR, Fernando FS, Montassier HJ, André MR, de Oliveira Vasconcelos R. Polarized M2 macrophages in dogs with visceral leishmaniasis. Vet Parasitol. 2016;226: 69–73. doi:10.1016/j.vetpar.2016.06.032
66. Mukherjee P, Sen PC, Ghose AC. Lymph node cells from BALB/c mice with chronic visceral leishmaniasis exhibiting cellular anergy and apoptosis: involvement of Ser/Thr phosphatase. Apoptosis. 2006;11: 2013–29. doi:10.1007/s10495-006-0088-7
67. Wu Z, Lu H, Sheng J, Li L. Inductive microRNA-21 impairs anti-mycobacterial responses by targeting IL-12 and Bcl-2. FEBS Lett. 2012;586: 2459–2467. doi:10.1016/j.febslet.2012.06.004
68. Cui B, Liu W, Wang X, Chen Y, Du Q, Zhao X, et al. Brucella Omp25 Upregulates miR-155, miR-21-5p, and miR-23b to Inhibit Interleukin-12 Production via Modulation of Programmed Death-1 Signaling in Human Monocyte/Macrophages. Front Immunol. 2017;8: 708. doi:10.3389/fimmu.2017.00708

7. SUPPORT INFORMATION

S1a Table. Complete blood count of dogs in the control group.

Animal	RBC	VG	Hemoglobin	VCM	CHCM
Reference value	5.5 - 8.5 $x10^6/\mu L$	37 – 55 %	12—18 g/dL	60 – 77 fL	32 – 36 %
Control 1	8.26	55	18	66.58	32.72
Control 2	7.21	53.8	18.8	74.7	34.9
Control 3	5.32	41.2	12.9	77.5	31.3
Control 4	7.43	56.7	20.4	76.4	35.9

RBC (red blood cells) CHCM (mean corpuscular hemoglobin concentration) MCV (mean corpuscular volume)

S1b Table. Complete blood count of dogs in the control group.

Animal	Wbc	Segmented	Lymphocyte	Monocyte	Eosinophil	Basophil	Plt	Tpp
Reference value	6–17 $x10^3/\mu L$	3.000-11.000/µL	1.000-4.800/µL	150-1.350/µL	150-1.250/µL	Raros/µL	160–430$x10^3/\mu L$	6.0-8.0 g/dL
Control 1	10.2	6.324	3.468	102	306	0	341	5.2
Control 2	13.1	6.419	6.668	174	261	0	341	5.2
Control 3	6.4	4.480	1.024	512	384	0	211	6

Control 4	10.6	7.844	1.060	424	1.250	0	228	7

TPP (total plasma protein)

S1c Table. Biochemical profile of control group dogs

Animal	Albumin	Uric Acid	ALT	AST	Creatinine	ALP	Total protein	Urea	GGT
Reference value	26 – 33 g/L	0 – 2 mg/dL	21 – 102 UI/L	23 – 66 UI/L	0.5 - 1.5 mg/dL	20 – 156 UI/L	54 – 71 g/L	1.67-8.33 mmol/L	1.2 - 6.4 UI/L
Control 1	32.87	1.31	71.86	20.83	1.1	30.26	59.37	8.46	2.91
Control 2	31.44	1.32	32.71	55.87	0.91	143.13	60.67	5.12	1.29
Control 3	34.03	2.18	34.3	24.73	4.36	41.49	56.87	6.43	2.62
Control 4	31.55	1.53	69.67	38.67	1.31	98.21	55.34	6.35	4.22

ALT (alanine aminotransferase) AST (aspartate aminotransferase) ALP (alkaline phosphatase) GGT (gamma glutamyl transferase)

S2 Tabela. Serological and molecular diagnosis of dogs of the control group.

Animal	**ELISA**	**PCR**
Control 1	0.026	Negative
Control 2	0.028	Negative
Control 3	0.049	Negative
Control 4	0.055	Negative

S3 Table. Main clinical signs associated with canine visceral leishmaniasis, serological and molecular diagnosis observed in dogs with visceral leishmaniasis.

Animal	**ELISA**	**PCR**	**Clinical Sign**
Infected 1	1.333	Positive	Onychogrifose, skin lesion, alopecia, ear lesion, anemia, hepatosplenomegaly
Infected 2	0.511	Positive	Onychogrifose, cachexia, skin lesion, alopecia, ear lesion, anemia, hepatosplenomegaly
Infected 3	0.974	Positive	Onychogrifose, cachexia, ear lesion, anemia
Infected 4	1.283	Positive	Onychogrifose, alopecia, ear injury, anemia
Infected 5	0.356	Positive	Lymphadenopathy, Onychogrifose, skin lesion, hepatosplenomegaly
Infected 6	1.323	Positive	Onychogrifose, skin lesion, ear lesion, anemia
Infected 7	1.315	Positive	Onychogrifosis, cachexia, skin lesion, ear lesion, anemia
Infected 8	0.907	Positive	Onychogrifose, cachexia, ear lesion, anemia, hepatosplenomegaly

S4a Table Biochemical profile of infected group dogs

Animal	Albumin	Uric acid	ALT	AST	Creatinine	FA	Total Protein	Urea	GGT
Reference value	26 – 33 g/L	0 – 2 mg/dL	21 – 102 UI/L	23 – 66 UI/L	0.5 - 1.5 mg/dL	20 – 156 UI/L	54 – 71 g/L	1.67-8.33 mmol/L	1.2 - 6.4 UI/L
Infected 1	8.18	0.29	22.02	21.45	1.08	34.38	45.16	19.42	0.94
Infected 2	14.01	0.63	32.44	93.56	0.73	55.43	89.2	4.54	2.3
Infected 3	14.65	0.28	29.97	31.89	1.24	92.16	65.33	13.89	5
Infected 4	10.36	0.83	25.08	34.49	0.74	121.25	54.81	7.33	3.24
Infected 5	25.57	0.44	15.42	30.12	0.84	42.96	62.42	6.46	1.93
Infected 6	14.77	0.84	13.28	29.08	0.53	50.18	79.65	5.68	2.2
Infected 7	10.34	0.93	17.41	25.08	0.64	36.15	68.44	3.87	1.36
Infected 8	5.45	0.21	9.89	14.39	0.52	83.52	41.5	2.25	1.02

ALT (Alanina aminotransferase) AST (aspartato aminotransferase) GGT (gama glutamil transferase)

S4b Table. Complete blood count of dogs of the infected group.

Animal	RBC	VG	Hemoglobin	VCM	CHCM
Reference value	5.5 - 8.5 $x10^6/\mu L$	37 – 55 %	12—18 g/dL	60 – 77 fL	32 – 36 %
Infected 1	5.05	33	11.5	65.35	34.85
Infected 2	4.91	31	11.1	63.14	35.81
Infected 3	3.41	25.4	8.6	74.5	25.2
Infected 4	5.27	34.5	10.4	65.6	30.1
Infected 5	4.24	31.3	10.6	74	33.8
Infected 6	4.93	31.2	10.3	63.4	33
Infected 7	4.23	33	10.3	63.5	33.1
Infected 8	5.28	34	10.6	60.2	32.4

RBC (red blood cells) CHCM (mean corpuscular hemoglobin concentration) MCV (mean corpuscular volume)

S4c Table. Complete blood count of dogs of the infected group.

Animal	Wbc	Segmented	Lymphocyte	Monocyte	Eosinophil	Basophile	Plt	Tpp
Reference value	6–17 $x10^3/\mu L$	3.000-11.000/µL	1.000 - 4.800/µL	150 - 1.350/µL	150 - 1.250/µL	Raros/µL	160–430 $x10^3/\mu L$	6.0-8.0 g/dL
Infected 1	12.5	9.500	1.375	1.125	500	0	420	9
Infected 2	11.8	10.738	708	354	0	0	60	10.9
Infected 3	10.2	7.752	1.836	510	102	0	82	9
Infected 4	6.3	4.158	1.071	756	63	0	159	6.8
Infected 5	14.8	6.808	5.032	2.220	740	0	168	11
Infected 6	14.9	9.238	3.427	1.043	1.192	0	277	10.2

Infected 7	12.5	10.000	1.750	375	375	0	331	8
Infected 8	9.8	6.272	2.156	392	980	0	145	9

TPP (total plasma protein)

S5 Table. Serological and molecular diagnosis of control dogs.

Animal	ELISA	PCR
Control 1	0.046	Negative
Control 2	0.047	Negative
Control 3	0.048	Negative
Control 4	0.056	Negative

S6 Tabela. Optical density on ELISA, PCR diagnostic and clinical signs of animals naturally infected dog from infected group

Animal	ELISA	PCR	Clinical Signs
Infected 1	1.044	Positive	Lymphadenopathy, Ear lesions, Periocular lesions
Infected 2	1.124	Positive	Lymphadenopathy, Onychogrifose, Ear lesions, Periocular lesions
Infected 3	1.030	Positive	Lymphoadenopathy, Onychogrifose, Ear lesions
Infected 4	0.479	Positive	Ear lesions, Periocular lesions, Ascites, Fucking lesion.
Infected 5	0.441	Positive	Lymphadenopathy, Ear lesions, Periocular lesions
Infected 6	1.103	Positive	Fracture Lesion, Lymphadenopathy, Ear Injuries
Infected 7	0.506	Positive	Lymphadenopathy, Hepatosplenomegaly, Injuries to the ear
Infected 8	0.924	Positive	Lymphadenopathy, Onychogrifose, Cachexia

S7a Table Complete blood count of dogs from the infected and control group

Animal	RBC	VG	Hemoglobina	VCM	CHCM
Reference value	5.5 - 8.5 x10^6/µL	37 – 55 %	12—18 g/dL	60 – 77 fL	32 – 36 %
Infected 1	4.64	29.0	9.4	62.5	32.4
Infected 2	4.45	32.3	11.2	72.7	34.6
Infected 3	5.82	32.5	10.7	55.9	32.9
Infected 4	2.39	15.5	5.2	65.0	33.5
Infected 5	2.99	18.4	5.6	61.6	30.4
Infected 6	6.52	52.2	18.1	80.2	34.6
Infected 7	6.31	45.7	16.3	72.5	35.6
Infected 8	5.94	35.6	11.7	60.0	32.8
Control 1	8.6	58.6	21.6	67.5	36.9
Control 2	8.2	56.3	20.6	66.1	36.5
Control 3	7.4	53.5	19.6	67.3	36.6
Control 4	5.77	40.5	14.7	70.2	36.4

RBC (red blood cells) CHCM (mean corpuscular hemoglobin concentration) MCV (mean corpuscular volume)

S7b Table. Complete blood count of infected and control dogs

Animal	Leukocytes	Neutrophils	Lymphocyte	Monocyte	Eosinophil	Plt
Reference value	6 – 17 x10^3/µL	3.000 - 11.000/µL	1.000 - 4.800/µL	150 - 1.350/µL	150 - 1.250/µL	160 – 430x10^3/µL
Infected 1	6.3	75	18	4	3	67
Infected 2	4.8	70	25	4	1	53
Infected 3	32.2	74	16	7	3	227
Infected 4	7.5	53	35	8	4	36
Infected 5	1.8	64	24	12	0	40
Infected 6	24.1	70	22	6	2	274
Infected 7	15.9	75	27	8	5	296
Infected 8	6.1	73	22	2	3	136
Control 1	14.26	6.702	4.535	528	2.481	201
Control 2	14.49	8.259	4.970	725	435	
Control 3	10.64	6.065	2.554	532	1.373	439
Control 4	20.22	11.222	5.540	849	2.608	386

TPP (total plasma protein)

S7c Table. Biochemical profile of infected and control group dogs

Animal	ALT	AST	Creatinine	ALP	Urea
Reference value	21 – 102 UI/L	23 – 66 UI/L	0.5 - 1.5 mg/dL	20 – 156 UI/L	1.67-8.33 mmol/L
Infected 1	15,05	63,88	3,30	116,09	39,79
Infected 2	52,92	56,83	0,57	113,3	4,31
Infected 3	102,85	67,11	0,59	101,71	7,18
Infected 4	99,03	68,01	0,83	185,19	6,55
Infected 5	31,39	75,53	1,03	148,84	6,15
Infected 6	34,70	35,56	0,73	91	6,48
Infected 7	26,39	88,22	0,59	79,66	4,56
Infected 8	24,06	62,64	0,98	0	6,90
Control 1	28.41	22.04	0.78	92.93	6.32
Control 2	35.82	34.56	1.12	143.09	7.1
Control 3	82.29	29.87	0.91	158.86	4.94
Control 4	89.19	40.77	0.91	70.53	4.95

ALT (Alanina aminotransferase) AST (aspartato aminotransferase)

S8 Table Efficiency and R^2 of the validation reactions of the SL miRs.

miRNA	**Eficiência**	**R^2**
miR 21	1,13	0,985
miR 148a	0,98	0,991
miR 7	1,03	0,985
miR 615	1,08	0,986
miR 150	1,02	0,947
miR 125a	0,91	0,992
miR 125b	1,0	0,994

S9 Table. Canonical pathways predicted for the miRNAs differentially regulated in CVL.

Ingenuity Canonical Pathways	**P-valor**
Neuregulin Signaling	0,000145
STAT3 Pathway	0,001
PTEN Signaling	0,001
HER-2 Signaling in Breast Cancer	0,001549
Myc Mediated Apoptosis Signaling	0,001549
Glioma Signaling	0,001862
Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells	0,001905
Neurotrophin/TRK Signaling	0,001905
ErbB Signaling	0,001905
Cholecystokinin/Gastrin-mediated Signaling	0,002188
Type I Diabetes Mellitus Signaling	0,002692
TGF-β Signaling	0,003236
VEGF Family Ligand-Receptor Interactions	0,003236

Molecular Mechanisms of Cancer	0,00389
Th2 Pathway	0,005623
VEGF Signaling	0,007079
Th1 and Th2 Activation Pathway	0,007079
Graft-versus-Host Disease Signaling	0,007079
p53 Signaling	0,007079
Prolactin Signaling	0,007079
Crosstalk between Dendritic Cells and Natural Killer Cells	0,007079
JAK/Stat Signaling	0,007079
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	0,007762
Virus Entry via Endocytic Pathways	0,008511
HGF Signaling	0,01
p38 MAPK Signaling	0,010233
ErbB2-ErbB3 Signaling	0,010471
Antigen Presentation Pathway	0,01122
Fc Epsilon RI Signaling	0,01122
NGF Signaling	0,01122
ErbB4 Signaling	0,01122
Axonal Guidance Signaling	0,01122
Prostate Cancer Signaling	0,012303
Renin-Angiotensin Signaling	0,012303
BMP signaling pathway	0,013804
Melanoma Signaling	0,014125
Non-Small Cell Lung Cancer Signaling	0,014125
Hepatic Fibrosis / Hepatic Stellate Cell Activation	0,014125
IL-6 Signaling	0,014791
Allograft Rejection Signaling	0,014791
SAPK/JNK Signaling	0,015488
Erythropoietin Signaling	0,016218
Chronic Myeloid Leukemia Signaling	0,016596
Renal Cell Carcinoma Signaling	0,016596
Role of NFAT in Cardiac Hypertrophy	0,016982
Aryl Hydrocarbon Receptor Signaling	0,017783
Cell Cycle: G1/S Checkpoint Regulation	0,017783
Human Embryonic Stem Cell Pluripotency	0,018621
FLT3 Signaling in Hematopoietic Progenitor Cells	0,018621
PEDF Signaling	0,018621
Death Receptor Signaling	0,018621
Autoimmune Thyroid Disease Signaling	0,018621
Xenobiotic Metabolism Signaling	0,018621
Paxillin Signaling	0,018621
Endometrial Cancer Signaling	0,018621
ERK/MAPK Signaling	0,018621
Bladder Cancer Signaling	0,018621
Insulin Receptor Signaling	0,018621
UVC-Induced MAPK Signaling	0,018621
Thrombopoietin Signaling	0,018621
IL-4 Signaling	0,018621
Apoptosis Signaling	0,019498
Hereditary Breast Cancer Signaling	0,019498
T Helper Cell Differentiation	0,020417
Breast Cancer Regulation by Stathmin1	0,020893

Neuroinflammation Signaling Pathway	0,023988
NF-κB Signaling	0,025704
VDR/RXR Activation	0,026303
PI3K/AKT Signaling	0,026915
Melanocyte Development and Pigmentation Signaling	0,026915
FAK Signaling	0,027542
Colorectal Cancer Metastasis Signaling	0,028184
Regulation of eIF4 and p70S6K Signaling	0,029512
PAK Signaling	0,029512
IL-15 Signaling	0,0302
CCR3 Signaling in Eosinophils	0,0302
p70S6K Signaling	0,033113
Glioblastoma Multiforme Signaling	0,033113
Protein Kinase A Signaling	0,033884
Small Cell Lung Cancer Signaling	0,033884
Mouse Embryonic Stem Cell Pluripotency	0,033884
IGF-1 Signaling	0,033884
Actin Cytoskeleton Signaling	0,033884
Inhibition of Matrix Metalloproteases	0,033884
Actin Nucleation by ARP-WASP Complex	0,035481
Macropinocytosis Signaling	0,035481
Oncostatin M Signaling	0,035481
IL-3 Signaling	0,038019
Acute Phase Response Signaling	0,038905
IL-12 Signaling and Production in Macrophages	0,038905
Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	0,038905
Cardiac Hypertrophy Signaling	0,038905
FGF Signaling	0,039811
IL-7 Signaling Pathway	0,039811
UVB-Induced MAPK Signaling	0,041687
G Beta Gamma Signaling	0,041687
Osteoarthritis Pathway	0,041687
LPS-stimulated MAPK Signaling	0,042658
Tumoricidal Function of Hepatic Natural Killer Cells	0,045709
CCR5 Signaling in Macrophages	0,045709
HIF1α Signaling	0,046774
PDGF Signaling	0,046774
CNTF Signaling	0,047863
IL-2 Signaling	0,047863
Thyroid Cancer Signaling	0,047863
Synaptic Long Term Potentiation	0,047863
Caveolar-mediated Endocytosis Signaling	0,048978
RAR Activation	0,048978
Ceramide Signaling	0,048978
Natural Killer Cell Signaling	0,048978
ERK5 Signaling	0,048978
MSP-RON Signaling Pathway	0,048978
Antiproliferative Role of TOB in T Cell Signaling	0,048978
Estrogen-mediated S-phase Entry	0,048978

S10a Tabel. Top 30 - GO Biological Process 2018

Index	Name	P-value	Adjusted p-value	Z-score	Combined score
1	positive regulation of protein phosphorylation (GO:0001934)	2.391e-9	0.000001316	-1.91	37.85
2	regulation of apoptotic process (GO:0042981)	9.482e-9	0.000003480	-1.79	33.04
3	cytokine-mediated signaling pathway (GO:0019221)	2.667e-11	2.937e-8	-1.35	32.76
4	transmembrane receptor protein tyrosine kinase signaling pathway (GO:0007169)	1.955e-8	0.000005067	-1.74	30.85
5	epidermal growth factor receptor signaling pathway (GO:0007173)	7.638e-7	0.00005947	-2.15	30.29
6	antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-independent (GO:0002480)	0.000002884	0.0001671	-2.31	29.46
7	regulation of cyclin-dependent protein kinase activity (GO:1904029)	6.604e-8	0.000009088	-1.72	28.43
8	artery development (GO:0060840)	0.00001610	0.0005909	-2.39	26.39
9	positive regulation of apoptotic process (GO:0043065)	2.772e-7	0.00002775	-1.68	25.33
10	negative regulation of cell junction assembly (GO:1901889)	0.00002682	0.0008204	-2.34	24.65
11	regulation of endothelial cell development (GO:1901550)	0.0001780	0.002702	-2.85	24.59
12	vascular endothelial growth factor receptor signaling pathway (GO:0048010)	6.267e-8	0.000009088	-1.44	23.94
13	ERBB signaling pathway (GO:0038127)	1.388e-7	0.00001528	-1.49	23.49
14	positive regulation of cell proliferation (GO:0008284)	4.189e-7	0.00003844	-1.55	22.76
15	outflow tract septum morphogenesis (GO:0003148)	0.00004141	0.001036	-2.25	22.73
16	extrinsic apoptotic signaling pathway (GO:0097191)	4.822e-8	0.000008848	-1.34	22.53

17	negative regulation of ERBB signaling pathway (GO:1901185)	0.000008489	0.0003462	-1.85	21.63
18	negative regulation of T-helper cell differentiation (GO:0045623)	0.0003039	0.003792	-2.67	21.58
19	axonogenesis (GO:0007409)	0.000004187	0.0002195	-1.69	20.87
20	negative regulation of cyclin-dependent protein serine/threonine kinase activity involved in G1/S transition of mitotic cell cycle (GO:0031658)	0.0002368	0.003303	-2.49	20.78
21	peripheral nervous system development (GO:0007422)	0.00004141	0.001036	-2.05	20.65
22	negative regulation of transcription by competitive promoter binding (GO:0010944)	0.0003792	0.004260	-2.60	20.45
23	activation of MAPKKK activity (GO:0000185)	0.0002368	0.003303	-2.43	20.29
24	transmembrane receptor protein serine/threonine kinase signaling pathway (GO:0007178)	0.00006830	0.001567	-2.09	20.06
25	regulation of cell proliferation (GO:0042127)	2.301e-8	0.000005067	-1.13	19.85
26	regulation of intracellular signal transduction (GO:1902531)	0.00003233	0.0008980	-1.91	19.79
27	regulation of myosin-light-chain-phosphatase activity (GO:0035507)	0.0002368	0.003303	-2.36	19.70
28	ventricular septum morphogenesis (GO:0060412)	0.00003608	0.0009690	-1.92	19.66
29	small GTPase mediated signal transduction (GO:0007264)	0.00001142	0.0004490	-1.72	19.61
30	ventricular septum development (GO:0003281)	0.00008437	0.001717	-2.07	19.42

S10b Tabel. Top 30 - GO Cellular Component 2018

Index	**Name**	**P-value**	**Adjusted p-value**	**Z-score**	**Combined score**
1	MHC protein complex (GO:0042611)	2.561e-7	0.00001895	-2.11	32.05

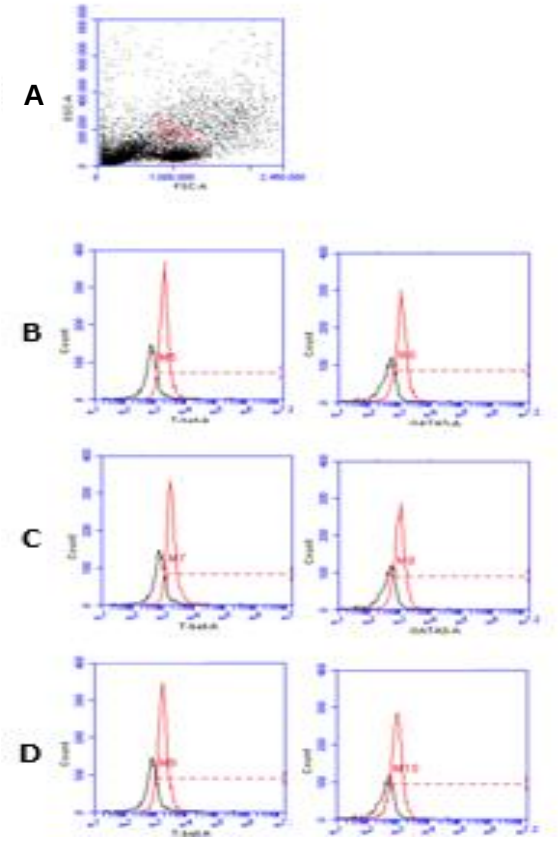
2	COPII-coated ER to Golgi transport vesicle (GO:0030134)	0.000002958	0.00005471	-2.10	26.73
3	integral component of lumenal side of endoplasmic reticulum membrane (GO:0071556)	0.000001767	0.00004359	-1.94	25.71
4	ER to Golgi transport vesicle membrane (GO:0012507)	5.838e-7	0.00002160	-1.76	25.26
5	axon (GO:0030424)	0.0008189	0.006733	-1.99	14.12
6	phagocytic vesicle membrane (GO:0030670)	0.0002066	0.003058	-1.56	13.22
7	integral component of endoplasmic reticulum membrane (GO:0030176)	0.0005714	0.005285	-1.75	13.04
8	spanning component of plasma membrane (GO:0044214)	0.02047	0.07973	-3.11	12.09
9	death-inducing signaling complex (GO:0031264)	0.02624	0.09248	-3.19	11.62
10	recycling endosome membrane (GO:0055038)	0.0003838	0.004597	-1.43	11.25
11	dendrite (GO:0030425)	0.003786	0.02155	-1.93	10.76
12	early endosome membrane (GO:0031901)	0.001362	0.01008	-1.54	10.13
13	recycling endosome (GO:0055037)	0.0004348	0.004597	-1.24	9.56
14	early endosome (GO:0005769)	0.004240	0.02241	-1.66	9.06
15	late endosome lumen (GO:0031906)	0.02912	0.09794	-2.50	8.85
16	phagocytic vesicle (GO:0045335)	0.001961	0.01319	-1.41	8.80
17	spanning component of membrane (GO:0089717)	0.03484	0.1121	-2.24	7.51
18	cytoplasmic vesicle (GO:0031410)	0.003786	0.02155	-1.26	7.03
19	MHC class II protein complex (GO:0042613)	0.04336	0.1284	-2.13	6.69
20	integral component of plasma membrane (GO:0005887)	0.009966	0.04609	-1.45	6.69
21	membrane raft (GO:0045121)	0.005363	0.02646	-1.28	6.68
22	SCF ubiquitin ligase complex (GO:0019005)	0.01149	0.05003	-1.48	6.63
23	caveola (GO:0005901)	0.01231	0.05059	-1.33	5.86
24	perinuclear region of cytoplasm (GO:0048471)	0.02552	0.09248	-1.53	5.62

25	serine/threonine protein kinase complex (GO:1902554)	0.07398	0.1781	-2.07	5.40
26	MLL1 complex (GO:0071339)	0.07944	0.1781	-2.08	5.27
27	endosome lumen (GO:0031904)	0.07124	0.1781	-1.94	5.13
28	MLL1/2 complex (GO:0044665)	0.07944	0.1781	-1.97	4.98
29	Golgi membrane (GO:0000139)	0.04162	0.1283	-1.47	4.67
30	contractile fiber (GO:0043292)	0.07672	0.1781	-1.80	4.61

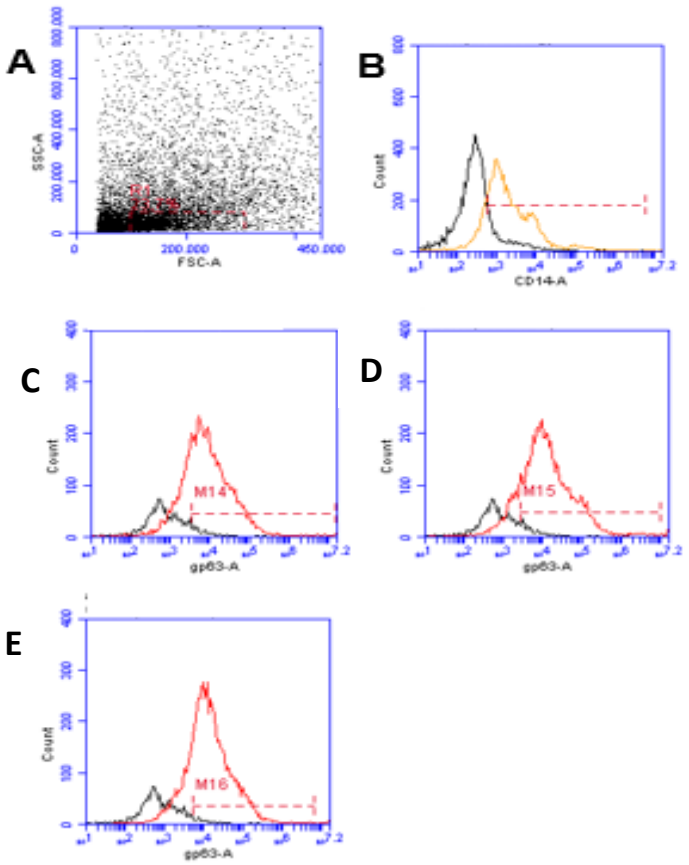
S10c Tabel. Top 30-GO Molecular Function 2018

Index	Name	P-value	Adjusted p-value	Z-score	Combined score
1	1-phosphatidylinositol-3-kinase regulator activity (GO:0046935)	0.0001780	0.002986	-3.33	28.79
2	vascular endothelial growth factor-activated receptor activity (GO:0005021)	0.0002368	0.003417	-3.12	26.08
3	phosphatidylinositol 3-kinase regulator activity (GO:0035014)	0.0003039	0.003417	-2.98	24.11
4	type I transforming growth factor beta receptor binding (GO:0034713)	0.0003039	0.003417	-2.82	22.81
5	protein kinase C activity (GO:0004697)	0.0004626	0.004657	-2.87	22.05
6	protein serine/threonine kinase activity (GO:0004674)	1.138e-7	0.00001719	-1.26	20.18
7	growth factor activity (GO:0008083)	0.000001964	0.0001176	-1.47	19.31
8	neurotrophin TRK receptor binding (GO:0005167)	0.0003039	0.003417	-2.34	18.95
9	transmembrane receptor protein serine/threonine kinase activity (GO:0004675)	0.0008764	0.007352	-2.45	17.28
10	kinase binding (GO:0019900)	0.00003022	0.0009127	-1.57	16.31
11	cyclin-dependent protein kinase activity (GO:0097472)	0.0001492	0.002817	-1.83	16.10
12	cyclin-dependent protein serine/threonine kinase activity (GO:0004693)	0.0001367	0.002817	-1.79	15.90
13	protein kinase activator activity (GO:0030295)	0.00002237	0.0008445	-1.44	15.45
14	protein serine/threonine kinase inhibitor activity (GO:0030291)	0.003765	0.02187	-2.60	14.53

15	protein kinase activity (GO:0004672)	0.000002337	0.0001176	-1.09	14.12
16	transforming growth factor beta receptor binding (GO:0005160)	0.0003168	0.003417	-1.74	14.05
17	BMP receptor activity (GO:0098821)	0.02047	0.07025	-3.48	13.53
18	MAP kinase kinase kinase activity (GO:0004709)	0.001645	0.01080	-2.07	13.29
19	interleukin-6 receptor binding (GO:0005138)	0.02336	0.07349	-3.33	12.50
20	transmembrane receptor protein tyrosine kinase activity (GO:0004714)	0.0008150	0.007352	-1.60	11.39
21	protein homodimerization activity (GO:0042803)	0.003314	0.02002	-1.99	11.39
22	activin-activated receptor activity (GO:0017002)	0.02336	0.07349	-3.01	11.31
23	transforming growth factor beta-activated receptor activity (GO:0005024)	0.02047	0.07025	-2.87	11.16
24	growth factor receptor binding (GO:0070851)	0.002621	0.01649	-1.88	11.16
25	Arp2/3 complex binding (GO:0071933)	0.02336	0.07349	-2.87	10.77
26	kinase activity (GO:0016301)	0.001424	0.009773	-1.60	10.48
27	inositol tetrakisphosphate phosphatase activity (GO:0052743)	0.02912	0.08296	-2.94	10.38
28	protein kinase binding (GO:0019901)	0.00009877	0.002486	-1.09	10.08
29	protein serine/threonine kinase activator activity (GO:0043539)	0.004009	0.02242	-1.81	9.97
30	neurotrophin TRKA receptor binding (GO:0005168)	0.02047	0.07025	-2.56	9.94



S11 Figure: Representative Histogram obtained from Flow Cytometric analysis of T-Bet (T1, FL1) and GATA3 transcription factors (T2, FL2) in LVC-positive dogs (n = 8) transfected with negative control of " mimic, miR 21 mimic, miR 21 inhibitor all with the presence of Hiperfect (miScript miRNA Mimic and Inhibitor Qiagen ™, USA) and cultured for 67h. Selected lymphocyte population (A), in the presence of the Mimic of miR-21 (B), in the presence of C-Mimic of miR-21 (C), in the presence of Inhibitor (D). Gate in R lymphoid cell marking, gate in M marking for T-bet and GATA3, red peak T-bet and GATA3 positive and peak black positive for their respective ISO-Controls.



S12 Figure 1: Representative histogram obtained from the CD-14 (T1, FL1) and gp63 (T2, FL2) -labelled flow cytometric analysis on splenic leukocytes from CVL -positive dogs (n = 8) transfected with negative mimic control , miR 21 mimic, miR 21 inhibitor and transfection control, all with the presence of Hiperfect (miScript miRNA Mimic and Inhibitor Qiagen ™, USA) were cultured for 67h. The population of macrophages (A), labeled with CD-14 for population selection, without any stimulus (control) (B), in the presence of Mimic of miR-21 (C), in the presence of presence of C-Mimic miR-21 (D) and in the presence of the Inhibitor miR-21 (E). Gate in R marking of macrophagic cells, gate in M marking for gp63 and CD-14, red peak gp63 positive, peak orange CD-14 positive and peak black positive for their respective ISO-Controls.

3.CAPITULO 3 - *DATA ARTICLE*

Data on differentially expressed in splenic leukocytes miRNAs in dogs with visceral leishmaniasis

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Abstract

This article contains data on differentially expressed miRNAs in splenic leukocytes (SL) of dogs naturally infected by *Leishmania* (*L.*) *infantum* compared to healthy dogs. In recent years, studies with miRNAs have shown that these molecules play a critical role in the regulation and function of immune response [1].Differentially expressed miRNAs were identified by microarray, validated by real time real-time PCR (qPCR). Targets and pathways were analyzed using the Ingenuity Pathway Analysis program.

Keywords: dog, *Leishmania infantum*, miRNA

[1] This article will be submitted to the journal " Data Brief ".

Specifications Table

Subject area	*Immunology*
More specific subject area	*Molecular Immunology in canine visceral leishmaniasis*
Type of data	*Table, figure*
How data was acquired	Splenic cell of dogs infected with *L. infantum* and healthy dogs were collected of the fragment of the spleen macerated, isolated and added to 10 ml of RPMI-1640 medium *(Sigma-Aldrich, St. Louis, MO, USA). DNA was extracted with DNeasy kit (Qiagen, Valencia, California, USA). Total RNA was extracted with MirVana kit with phenol (Life Technologies, USA), microarray was performed using GeneAtlas® Hybridization, Wash, and Stain Kit (Affymetrix, USA). Validation was done by qPCR and analysis in the Ingenuity Pathway Analysis (Qiagen®).*
Data format	*Analysed*
Experimental factors	*Leishmania infantum specie was identified by PCR-RFLP*
Experimental features	*Total RNA was extracted from 5x10^{6}LE of dogs infected with L. infantum and healthy dogs. Microarray was performed using miRNAs with RNA Integrity Number greater than 8.*
Data source location	*São Paulo State University, Araçatuba, São Paulo, Brazil.*
Data accessibility	*Microarray data were deposited in Gene Expression Omnibus with the access number GSE112459*

Value of the data

- **MicroRNA profile spleen leukocytes in dogs with VL was described for the first time.**
- ***Leishmania infantum* infection differentially regulates miRNA expression.**
- **Due to reemergence of VL, miRNA data obtained herein is fundamental to disease control.**

1. Data

Data shown in this article provide information about miRNAs differentially expressed in SL of dogs with VL compared to healthy dogs. Total RNA was extracted from SL of healthy dogs (control group) and infected dogs (infected group). Control group was composed by five healthy dogs of different breeds, weight and age between

1 and 5 years. Infected group was composed by ten dogs naturally infected with *L. infantum* with at least 3 characteristic symptoms of CVL, housed in Araçatuba, an endemic region in Brazil. All animals were of different breeds, weight and age between 1 and 5 years. Physical examination, complete blood count and serum biochemical profile were performed. ELISA was employed to confirm the presence or absence of anti-*leishmania* antibodies (Table 1a and b).

Table 1a. Optical density on ELISA and clinical signs of animals naturally infected

Animal	ELISA	Clinical Sign
Infected 1	1.333	Onychogrifose, skin lesion, alopecia, ear lesion, anemia, hepatosplenomegaly
Infected 2	0.511	Onychogrifose, cachexia, skin lesion, alopecia, ear lesion, anemia, hepatosplenomegaly
Infected 3	0.974	Onychogrifose, cachexia, ear lesion, anemia
Infected 4	1.283	Onychogrifose, alopecia, ear injury, anemia
Infected 5	0.356	Lymphadenopathy, Onychogrifose, skin lesion, hepatosplenomegaly
Infected 6	1.323	Onychogrifose, skin lesion, ear lesion, anemia
Infected 7	1.315	Onychogrifosis, cachexia, skin lesion, ear lesion, anemia
Infected 8	0.907	Onychogrifose, cachexia, ear lesion, anemia, hepatosplenomegaly

Table 1b. Optical density on ELISA of animals control

Animal	ELISA
Control 1	0.046
Control 2	0.047
Control 3	0.048
Control 4	0.056

2. Experimental Design, Materials and Methods

2.1. Animal screening and collection of samples

This study was approved by the Brazilian Council on Animal Experimentation (COBEA), with the approval of the Committee for Ethics in Animal Use (CEUA) of UNESP – Universidade Estadual Paulista "Júlio de Mesquita Filho" - Campus de Araçatuba - School of Veterinary Medicine - FMVA (process 00978/2016).

Four healthy animals upon clinical examination, complete blood count and serum biochemical profile were selected for this study, and eight animals naturally infected with *Leishmania infantum,* from the Zoonosis Control Center of Araçatuba, were selected. These animals contained at least three characteristic clinical signs of the disease, including onychogriphosis, weight loss, ear-tip injuries, periocular lesions, alopecia, skin lesions and lymphadenopathy. Infected animals were classified in the clinical stage II of the disease [2]. Blood from both groups (infected and control) was collected in tubes without EDTA to obtain serum for biochemical profile and perform indirect ELISA assay for the detection of anti-leishmania antibodies [3], and in EDTA tubes for hemogram and isolation of the PBMC. Infected animals were euthanized by barbiturate anesthesia (Tiopental, Cristália Itapira, SP), followed by intravenous injection of potassium chloride 19.1% by the same route, in compliance with local laws.

2.2. Isolation of splenic leukocytes

Total SL were obtained from a $2cm^3$ fragment that was macerated and added to 10ml RPMI-1640 medium (Sigma®, USA) supplemented with 10% heat inactivated fetal bovine serum, 0.03% L- glutamine, and 100 IU / mL penicillin and 100 mg / mL streptomycin. After removal of the cell debris through a 100µm filter (BD Falcon ™ Cell strainer, USA), the suspension was processed with 5 mL of red blood cell lysis buffer containing 7.46 g / L ammonium chloride (NH 4 ClO 3) at 4 For 10 minutes, centrifuged at 2000 rpm for 5 minutes and washed with phosphate buffered saline at pH 7.2 for three times. After obtaining these cells, they were counted in Neubauer's chamber for later extraction of DNA and miRNA.

2.3. DNA extraction and determination of the *Leishmania* species

DNA extraction from SL samples from the experimental dogs was performed using $5x10^6$ cells with the commercial DNAeasy® kit (Qiagen, Valencia, California, USA) according to manufacturer's recommendations. Extracted DNA was quantified in spectrophotometer 260/280 (NanoDrop, Thermo Fisher Scientific) for evaluation of purity and then stored at -20°C until analysis.

Determination of *Leishmania* species was performed by PCR-RFLP (Restriction Fragment Length Polymorphism) [4], comparing the restriction profiles of the sample with a PCR restriction profile obtained from *L. infantum* (IOC / L0575-MHOM / BR / 2002 / LPC-RPV), *L. braziliensis* (IOC / L0566-MHOM / BR / 1975 / M2903) and *L. amazonensis* (IOC / L0575-MHOM / BR / 1967 / PH8) as positive controls and water as negative control.

2.4. Extraction and quantification of Total RNA

Extraction of total RNA from SL was performed on the samples with the commercial mirVana® kit for isolation with phenol (Life Technologies, USA), which preserves small RNAs, following procedure indicated by the manufacturer. After isolation, total RNA was stored at -80ºC until evaluation of quality and concentration.

Isolated RNAs were analyzed by spectrophotometry (NanoDrop, Thermo Fisher Scientific) for evaluation of their quantity (260/280). Before performing microarray, samples were also analyzed for RNA quality by capillary electrophoresis (Bioanalyzer, Agilent Technologies, USA) using the commercial Agilent RNA 6000 Nano kit, following manufacturer's instructions (Fig. 1).

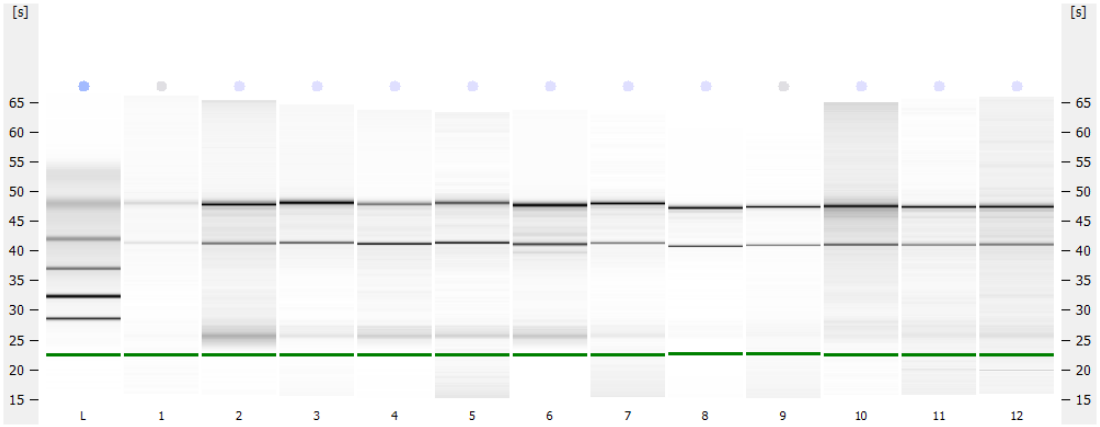

Fig. 1. **Quality of miRNA.** Bioanalyzer gel image of RNA samples used for microarray analysis. All samples used had RNA integrity numbers above 8.

2.5. Microarray

Total RNA samples with concentration 250ng/µl and quality (RIN>8) were used to perform microarray analysis using a miRNA 4.1 Array Strip (Affymetrix, USA), containing probes designed for the miRNAs of several species, including 291 of *Canis familiaris*.

MicroRNAs were biotinylated using the Affymetrix FlashTag™ Biotin HSR RNA Labeling Kit following manufacturer's instructions. For the microarray, GeneAtlas® Hybridization, Wash, and Stain Kit for miRNA array Strips were used, following manufacturer's instructions.

Microarray data were deposited in the Gene Expression Omnibus with the access number GSE112459 according to the minimum information about microarray experiment (MIAME) standards.

2.6. Microarray data analysis

Normalization and quality of microarray analysis of the miRNAs of control and infected dogs were performed in the Expression Console Software program, version 1.4.1 (Affymetrix, Thermo Fisher Scientific, USA) (Fig. 2). Differential analysis of the miRNAs was performed in the Transcriptome Analysis Console (Affymetrix, Thermo Fisher Scientific, USA) (Fig. 3).

Analysis of targets and pathways of the miRNAs differentially expressed in dogs with VL were performed using the Ingenuity Pathway Analysis (Qiagen, Redwood City, CA, USA).

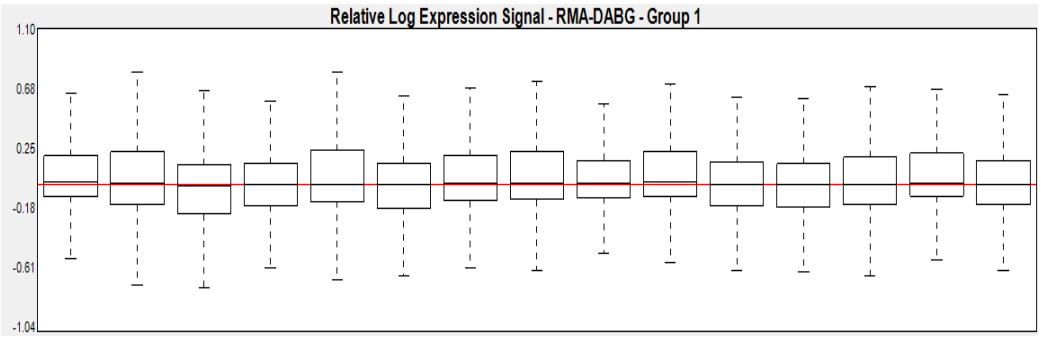

Fig. 2. Values of relative log expression signal (RMA-DABG normalized) between groups

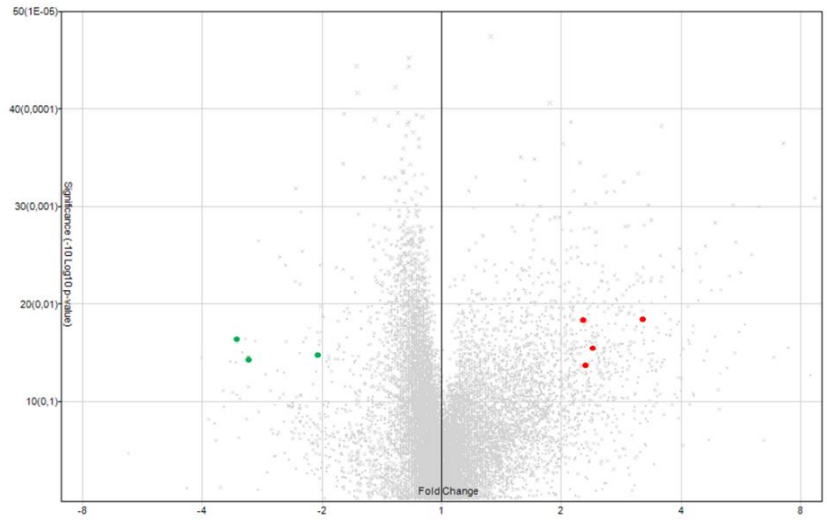

Fig. 3. Distribution of differentially expressed miRNAs. Upregulated (red) and downregulated (green) miRNAs in SL of dogs infected with *L. infantum* compared to healthy dogs. The canine miRNAs hybridized to the microarray with probes of other species (mammals, birds, fish, plants), but none of them showed homology with the currently available canine sequences currently known (gray).

2.7. Real-time PCR for miRNA analysis

To validate the results obtained by microarray, qPCR was performed. cDNA production was performed using the miScript RT II kit (Qiagen™), as recommended by the manufacturer. qPCR reactions were performed using commercially available specific primers for dog miRNAs of interest and reference genes (miScript, Qiagen™) using the SYBR Green system (myScript SYBR Green PCR kit, Qiagen™) in the real-time thermal cycler (RealPlex, Eppendorf™). Amplification conditions were determined by the manufacturer. For each reaction, a standard curve with serial dilution of a pool of cDNAs was performed. MicroRNA expression was calculated based on the values of the standard curve, by the quotient of the target miRNA value and the housekeeping gene value.

2.8. Statistical analysis

Statistical analyses were performed using the GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). Analysis of variance (ANOVA) was

performed for treatment comparison in the microarray. Results were considered significant when $p < 0.05$.

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References

[1] Liu, G.; Abraham, E. (2013). **MicroRNAs in immune response and macrophage polarization. Arteriosclerosis, Thrombosis, and Vascular Biology**. DOI: 10.1161/ATVBAHA.112.300068

[2] Solano-Gallego, L., Koutinas, A., Miró, G., Cardoso, L., Pennisi, M.G., Ferrer, L., Bourdeau, P., Oliva, G., Baneth, G.(2009). **Directions for the diagnosis, clinical staging, treatment and prevention of canine leishmaniosis**. Veterinary Parasitology. DOI:10.1016/j.vetpar.2009.05.022

[3] Lima, V.M.F., Gonçalves, M.E., Ikeda, F.A., Luvizotto, M.C.R., Feitosa, M.M. (2003). **Anti-leishmania antibodies in cerebrospinal fluid from dogs with visceral leishmaniasis**. Brazilian Journal of Medical and Biological Research.DOI: 10.1590/S0100-879X2003000400010

[4] Sanches, L. da C., Martini, C.C. de, Nakamura, A.A., Santiago, M.E.B., Dolabela de Lima, B., Lima, V.M.F. de. (2016). **Natural canine infection by Leishmania infantum and Leishmania amazonensis and their implications for disease control**. Revista Brasileira de Parasitologia Veterinária DOI:10.1590/s1984-29612016071

[5] Ranasinghe, S., Rogers, M.E., Hamilton, J.G.C., Bates, P.A., Maingon, R.D.C. (2008). **A real-time PCR assay to estimate Leishmania chagasi load in its natural sand fly vector Lutzomyia longipalpis**. Transaction of the Royal Society of Tropical Medicine and Hygiene. DOI:10.1016/j.trstmh.2008.04.003

APÊNDICE- REFERÊNCIAS DA INTRODUÇÃO GERAL

AKHOUNDI, M. et al. A Historical Overview of the Classification, Evolution, and Dispersion of Leishmania Parasites and Sandflies. **PLOS Neglected Tropical Diseases**, v. 10, n. 3, p. e0004349, 3 mar. 2016.

ALEXANDRE-PIRES, G. et al. Leishmaniosis—A report about the microvascular and cellular architecture of the infected spleen inCanis familiaris. **Microscopy Research and Technique**, v. 69, n. 4, p. 227–235, abr. 2006.

ALVAR, J. et al. Leishmaniasis Worldwide and Global Estimates of Its Incidence. **PLoS ONE**, v. 7, n. 5, p. e35671, 31 maio 2012.

BANETH, G. et al. Canine leishmaniosis - new concepts and insights on an expanding zoonosis: part one. **Trends in parasitology**, v. 24, n. 7, p. 324–30, 1 jul. 2008.

BELOSEVIC, M. et al. IL-2. A cofactor for induction of activated macrophage resistance to infection. **Journal of immunology (Baltimore, Md. : 1950)**, v. 145, n. 3, p. 831–9, 1 ago. 1990.

BHATTACHARYA, P. et al. Identification of microRNA-21 as a biomarker in live attenuated Leishmania vaccine induced protective immunity. **The Journal of Immunology**, v. 198, n. 1 Supplement, 2017.

BOURDOISEAU, G. et al. Lymphocyte subset abnormalities in canine leishmaniasis. **Veterinary immunology and immunopathology**, v. 56, n. 3–4, p. 345–51, maio 1997.

CABRAL, M.; O'GRADY, J.; ALEXANDER, J. Demonstration of Leishmania specific cell mediated and humoral immunity in asymptomatic dogs. **Parasite immunology**, v. 14, n. 5, p. 531–9, set. 1992.

CARISSIMI, C. et al. miR-21 is a negative modulator of T-cell activation. **Biochimie**, v. 107, p. 319–326, 1 dez. 2014.

CAVALCANTI, A. S. et al. Parasite Load Induces Progressive Spleen Architecture Breakage and Impairs Cytokine mRNA Expression in Leishmania infantum-Naturally Infected Dogs. **PLOS ONE**, v. 10, n. 4, p. e0123009, 13 abr. 2015.

CILLARI, E. et al. Reduction in the number of UCHL-1+ cells and IL-2 production in the peripheral blood of patients with visceral leishmaniasis. **Journal of immunology (Baltimore, Md. : 1950)**, v. 146, n. 3, p. 1026–30, 1 fev. 1991.

COUTINHO, M. T. Z. et al. Participation of Rhipicephalus sanguineus (Acari: Ixodidae)

in the epidemiology of canine visceral leishmaniasis. **Veterinary Parasitology**, v. 128, n. 1–2, p. 149–155, 10 mar. 2005.

COUTINHO, M. T. Z.; LINARDI, P. M. Can fleas from dogs infected with canine visceral leishmaniasis transfer the infection to other mammals? **Veterinary parasitology**, v. 147, n. 3–4, p. 320–5, 20 jul. 2007.

DANTAS-TORRES, F. Situação atual da epidemiologia da leishmaniose visceral em Pernambuco. **Revista de Saúde Pública**, v. 40, n. 3, p. 537–541, jun. 2006.

DE LIMA, V. M. F. et al. Apoptosis in T lymphocytes from spleen tissue and peripheral blood of L. (L.) chagasi naturally infected dogs. **Veterinary parasitology**, v. 184, n. 2–4, p. 147–53, 23 mar. 2012.

DESJEUX, P. Leishmaniasis: current situation and new perspectives. **Comparative Immunology, Microbiology and Infectious Diseases**, v. 27, n. 5, p. 305–318, set. 2004.

DIAZ, S. et al. Canine leishmaniosis. Modulation of macrophage/lymphocyte interactions by L. infantum. **Veterinary parasitology**, v. 189, n. 2–4, p. 137–44, 26 out. 2012.

DIOTALLEVI, A. et al. Leishmania Infection Induces MicroRNA hsa-miR-346 in Human Cell Line-Derived Macrophages. **Frontiers in Microbiology**, v. 9, p. 1019, 17 maio 2018.

DOS-SANTOS, W. L. C. et al. Associations among immunological, parasitological and clinical parameters in canine visceral leishmaniasis: Emaciation, spleen parasitism, specific antibodies and leishmanin skin test reaction. **Veterinary immunology and immunopathology**, v. 123, n. 3–4, p. 251–9, 15 jun. 2008.

EL GAZZAR, M. et al. MicroRNA-146a regulates both transcription silencing and translation disruption of TNF-α during TLR4-induced gene reprogramming. **Journal of leukocyte biology**, v. 90, n. 3, p. 509–19, set. 2011.

FEIJÓ, D. et al. Dendritic Cells and *Leishmania* Infection: Adding Layers of Complexity to a Complex Disease. **Journal of Immunology Research**, v. 2016, p. 1–9, 19 jan. 2016.

FERREIRA, M. G. P. A. et al. Potential role for dog fleas in the cycle of Leishmania spp. **Veterinary Parasitology**, v. 165, n. 1–2, p. 150–154, 28 out. 2009.

FISA, R. et al. Epidemiology of canine leishmaniosis in Catalonia (Spain) The example of the Priorat focus. **Veterinary Parasitology**, v. 83, p. 87–97, 1999.

FIUZA, J. A. et al. Vaccination using live attenuated Leishmania donovani centrin

deleted parasites induces protection in dogs against Leishmania infantum. **Vaccine**, v. 33, n. 2, p. 280–8, 3 jan. 2015.

GENARO, O. Leishmaniose visceral canina experimental. 1993.

GERACI, N. S.; TAN, J. C.; MCDOWELL, M. A. Characterization of microRNA expression profiles in Leishmania-infected human phagocytes. **Parasite immunology**, v. 37, n. 1, p. 43–51, jan. 2015.

GHOSH, J. et al. Leishmania donovani targets Dicer1 to downregulate miR-122, lower serum cholesterol, and facilitate murine liver infection. **Cell host & microbe**, v. 13, n. 3, p. 277–88, 13 mar. 2013.

GONTIJO, C. M. F.; MELO, M. N. Leishmaniose visceral no Brasil: quadro atual, desafios e perspectivas. **Revista Brasileira de Epidemiologia**, v. 7, n. 3, p. 338–349, set. 2004.

GRAFF, J. W. et al. Identifying functional microRNAs in macrophages with polarized phenotypes. **The Journal of biological chemistry**, v. 287, n. 26, p. 21816–25, 22 jun. 2012.

GREENWALD, R. J.; FREEMAN, G. J.; SHARPE, A. H. THE B7 FAMILY REVISITED. **Annual Review of Immunology**, v. 23, n. 1, p. 515–548, abr. 2005.

HUSEIN, A. et al. *Leishmania donovani* infection differentially regulates small G-proteins. **Journal of Cellular Biochemistry**, v. 119, n. 9, p. 7844–7854, set. 2018.

JIANG, A. et al. miR-615-3p promotes the phagocytic capacity of splenic macrophages by targeting ligand-dependent nuclear receptor corepressor in cirrhosis-related portal hypertension. **Experimental biology and medicine (Maywood, N.J.)**, v. 236, n. 6, p. 672–80, 1 jun. 2011.

LIU, G.; ABRAHAM, E. MicroRNAs in Immune Response and Macrophage Polarization. **Arteriosclerosis, Thrombosis, and Vascular Biology**, v. 33, n. 2, p. 170–177, fev. 2013.

MANCIANTI, F. et al. Studies on canine leishmaniasis control. 1. Evolution of infection of different clinical forms of canine leishmaniasis following antimonial treatment. **Transactions of the Royal Society of Tropical Medicine and Hygiene**, v. 82, n. 4, p. 566–7, 1988.

MOLYNEUX, D. H.; ASHFORD, R. W. The biology of Trypanosoma and Leishmania, parasites of man and domestic animals. **The biology of Trypanosoma and Leishmania, parasites of man and domestic animals.**, 1983.

MORENO, J.; ALVAR, J. Canine leishmaniasis: Epidemiological risk and the

experimental model. **Trends in Parasitology**, v. 18, n. 9, p. 399–405, 2002.

MUKHERJEE, B. et al. Antimony-Resistant *Leishmania donovani* Exploits miR-466i To Deactivate Host MyD88 for Regulating IL-10/IL-12 Levels during Early Hours of Infection. **The Journal of Immunology**, v. 195, n. 6, p. 2731–2742, 15 set. 2015.

NOLI, C.; BOOTHE, D. Macrolides and lincosamides. **Veterinary Dermatology**, v. 10, n. 3, p. 217–223, 1 set. 1999.

OWENS, S. D. et al. Transmission of visceral leishmaniasis through blood transfusions from infected English foxhounds to anemic dogs. **Journal of the American Veterinary Medical Association**, v. 219, n. 8, p. 1076–83, 15 out. 2001.

PANDEY, R. K.; SUNDAR, S.; PRAJAPATI, V. K. Differential expression of miRNA regulates T cell differentiation and plasticity during visceral leishmaniasis infection. **Frontiers in Microbiology**, v. 7, n. FEB, p. 1–9, 2016.

PENTCHEVA-HOANG, T. et al. Programmed death-1 concentration at the immunological synapse is determined by ligand affinity and availability. **Proceedings of the National Academy of Sciences**, v. 104, n. 45, p. 17765–17770, 6 nov. 2007.

PINELLI, E. et al. Cellular and humoral immune responses in dogs experimentally and naturally infected with Leishmania infantum. **Infection and immunity**, v. 62, n. 1, p. 229–35, jan. 1994.

PINELLI, E. et al. Leishmania infantum-specific T cell lines derived from asymptomatic dogs that lyse infected macrophages in a major histocompatibility complex-restricted manner. **European journal of immunology**, v. 25, n. 6, p. 1594–600, jun. 1995.

PINELLI, E. et al. Compensation for Decreased Expression of B7 Molecules on Leishmania infantum-Infected Canine Macrophages Results in Restoration of Parasite-Specific T-Cell Proliferation and Gamma Interferon Production. **INFECTION AND IMMUNITY**, v. 67, n. 1, p. 237–243, 1999.

QUEIROZ, P. V. S. et al. Canine visceral leishmaniasis in urban and rural areas of Northeast Brazil. **Research in Veterinary Science**, v. 86, n. 2, p. 267–273, abr. 2009.

REIS, A. B. et al. Systemic and compartmentalized immune response in canine visceral leishmaniasis. **Veterinary immunology and immunopathology**, v. 128, n. 1–3, p. 87–95, 15 mar. 2009.

SADICK, M. D. et al. Cytokine regulation of murine leishmaniasis: interleukin 4 is not sufficient to mediate progressive disease in resistant C57BL/6 mice. **Infection and immunity**, v. 59, n. 12, p. 4710–4, dez. 1991.

SANTA ROSA I.C.A.; OLIVEIRA I.C.S. Leishmaniose visceral: breve revisão sobre

uma zoonose reemergente. **Clinica Veterinária,** v. 2, n. 11, p. 24-28, 1997.

SANTANA, C. C. et al. Inflammation and structural changes of splenic lymphoid tissue in visceral leishmaniasis: a study on naturally infected dogs. **Parasite immunology**, v. 30, n. 10, p. 515–24, out. 2008.

SANTIAGO, M. E. B. et al. Improvement in clinical signs and cellular immunity of dogs with visceral leishmaniasis using the immunomodulator P-MAPA. **Acta Tropica**, v. 127, n. 3, p. 174–180, set. 2013.

SEMIÃO-SANTOS, S. J. et al. Evora district as a new focus for canine leishmaniasis in Portugal. **Parasitology research**, v. 81, n. 3, p. 235–9, 1995.

SLAPPENDEL, R. J.; FERRER, L. Leishmaniasis. In: GREENE, C.E. **Clinical microbiology and infectious diseases of the dog and cat.** Philadelphia: W.B. Saunders, p. 450-458, 1990.

STRAUSS-AYALI, D.; BANETH, G.; JAFFE, C. L. Splenic immune responses during canine visceral leishmaniasis. **Veterinary Research**, v. 38, n. 4, p. 547–564, 30 jul. 2007.

TIWARI, N. et al. Identification and Characterization of miRNAs in Response to Leishmania donovani Infection: Delineation of Their Roles in Macrophage Dysfunction. **Frontiers in microbiology**, v. 8, p. 314, 2017.

UCHINO, K.; OCHIYA, T.; TAKESHITA, F. RNAi Therapeutics and Applications of MicroRNAs in Cancer Treatment. **Japanese Journal of Clinical Oncology**, v. 43, n. 6, p. 596–607, jun. 2013.

VERMA, J. K.; RASTOGI, R.; MUKHOPADHYAY, A. Leishmania donovani resides in modified early endosomes by upregulating Rab5a expression via the downregulation of miR-494. **PLOS Pathogens**, v. 13, n. 6, p. e1006459, 26 jun. 2017.

WORLD HEALTH ORGANIZATION. **Leishmaniasis**. Disponível em: <http://www.who.int/en/news-room/fact-sheets/detail/leishmaniasis>. Acesso em: 29 out. 2018.